

IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE* SEROTYPES

FIELD OF THE INVENTION

The present invention relates to molecular methods of serotyping *Streptococcus pneumoniae*, as well as polynucleotides useful in such methods.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is a leading cause of morbidity and mortality causing invasive disease such as meningitis and pneumonia as well as more localised disease such as acute otitis media and sinusitis. Polysaccharide and protein-conjugate pneumococcal vaccines have the potential to prevent a significant proportion of cases. Effective protein-conjugate vaccines are particularly important because of the dramatic increase in prevalence and international dissemination of antibiotic resistant *S. pneumoniae* serotypes that commonly cause invasive disease in children (Hausdorff et al., 2001; Huebner, et al., 2000). However these vaccines protect against only the relatively small minority (Dunne et al., 2001; Hausdorff et al., 2001) of pneumococcal serotypes that most commonly cause disease. There is theoretical and limited empirical evidence that widespread use of these vaccines could lead to substitution of "vaccine" serotypes with other nonvaccine serotypes, against which the vaccines do not provide protection. Continued surveillance will be critical to monitor vaccine efficacy and changes in incidence and distribution of colonising and invasive serotypes (Hausdorff et al., 2001; Rubins et al., 1999). Any increase in disease caused by previously uncommon nonvaccine serotypes could necessitate a change in vaccine composition (Lipsitch, 2001).

S. pneumoniae comprises at least 90 serotypes, distinguished by capsular polysaccharide antigens. Pneumococcal serotype/group identification is currently performed, using large panels of expensive antisera, by various methods, including capsular swelling (Quellung) reaction - the traditional "gold standard" - latex agglutination and coagglutination (Arai et al., 2001; Lalitha et al., 1999). Cross-reactions between serotypes and discrepancies between methods can occur and some strains are nonserotypable (Henrichsen, 1999).

The capsular polysaccharide synthesis (*cps*) gene clusters for at least 16 pneumococcal serotypes have been sequenced and serotype-specific genes identified (Jiang et al., 2001; van Selm et al., 2002). The *cps* gene cluster contains genes responsible for synthesis of the serotype-specific polysaccharide including - except in serotype 3 - *wzy* (polysaccharide polymerase gene) and *wzx* (polysaccharide flippase

gene). At the 5'-end of the *cps* gene cluster are four relatively conserved open reading frames - *cpsA* (*wzg*)-*cpsB* (*wzh*)-*cpsC* (*wzd*)-*cpsD* (*wze*). Sequence differences in this region were used to classify 11 *S. pneumoniae* serotypes into two classes and, in the region between the 3'-end of *cpsA* and the 5'-end of *cpsB*, there were sites of
5 heterogeneity between and within serotypes (Jiang et al., 2001; Lawrence et al., 2000). *S. pneumoniae* is characterised by high frequency recombination within the *cps* gene cluster, leading to serotype "switching" among isolates within genetic lineages defined by relationships between their more conserved housekeeping genes (Coffey et al., 1998; Jiang et al., 2001).

10 The relatively low percentage of polymorphisms between strains which is linked to actual serotype, and the large number of different serotypes, has made the development of assays which can be used for typing a significant portion of *S. pneumoniae* strains difficult. Accordingly, there is a need for further methods which can be used to identify different *Streptococcus pneumoniae* serotypes.

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SUMMARY OF THE INVENTION

Through the complex analysis of a large number of polymorphisms which exist between at least 132 molecular capsular sequence types of *Streptococcus pneumoniae* the present inventors have devised methods which can be used to distinguish between a
20 majority of different *S. pneumoniae* serotypes. In particular, prior art methods of nucleic acid based typing techniques could serotype only about 20 serotypes of *S. pneumoniae*. In contrast, the methods of the invention can be used to serotype most of the about 90 serotypes of *S. pneumoniae*. The methods of the invention can also be used to subtype some serotypes.

25 Thus, in a first aspect, the present invention provides a method of distinguishing between at least 25 different serotypes of *Streptococcus pneumoniae* in a sample, the method comprising,

i) analysing at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, and/or

30 ii) analysing at least a portion of the *wzy* and/or *wzx* gene(s).

Preferably, the method can be used to type at least 40, more preferably at least 50, more preferably at least 70, more preferably at least 90, more preferably at least 100, even more preferably at least about 132 different molecular capsular sequence types of *S. pneumoniae*.

35 The present inventors are the first to provide suitable nucleic acid based techniques for typing a large number of *Streptococcus pneumoniae* serotypes.

Accordingly, in another aspect the present invention provides a method of determining the serotype of *Streptococcus pneumoniae* in a sample, the method comprising,

i) analysing at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, and/or

5 ii) analysing at least a portion of the *wzy* and/or *wzx* gene(s),

wherein the serotype is selected from the group consisting of: 2, 7A, 7B, 7C, 9A, 9L, 10F, 10A, 10B, 10C, 11F, 11A, 11B, 11C, 11D, 12F, 12A, 12B, 13, 15F, 15A, 15B, 15C, 16A, 17F, 17A, 18F, 18A, 18B, 21, 22F, 22A, 24F, 24A, 24B, 25F, 25A, 27, 28F, 28A, 31, 32F, 32A, 33F, 33A, 33B, 33C, 33D, 34, 35A, 35B, 35C, 36, 37, 38, 39, 40, 10 41F, 41A, 42, 43, 44, 45, 46, 47, 47A and 48.

The present inventors have surprisingly found that at least about 102 molecular capsular sequence types of *S. pneumoniae* can be directly serotyped by analysing the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene of the *S. pneumoniae* genome.

Thus, in another aspect the present invention provides a method of determining
15 the serotype of *Streptococcus pneumoniae* in a sample, the method comprising analysing at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene.

In a preferred embodiment, the portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene which is analysed is any
20 nucleotide which is polymorphic between at least some of the *S. pneumoniae* serotypes referred to in Figure 2.

In a particularly preferred embodiment, the method comprises amplifying at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, and sequencing the amplification product. More preferably,
25 the entire approximate 800 bp region as provided in Figure 2 is amplified and sequenced.

In the case of sequencing to identify the serotype, the sequencing primers are selected such that they hybridise specifically to a region within or near to a region within which a polymorphism is present. The primers need not be specific to particular
30 serotypes since it is the actual sequence information obtained during the sequencing process which is used to determine the *S. pneumoniae* serotype. Thus the primers may hybridise specifically to genomic DNA from all *S. pneumoniae* serotypes (or at least those serotypes referred to in Figure 2), or to genomic DNA from some, but not all, *S. pneumoniae* serotypes.

35 When a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene is amplified, it is preferable that the amplification is

performed using primer pairs comprising a sequence selected from the group consisting of:

- 1) GGCATT(/C)TATGGAGTTGATTCTG(/A)TCCATT(/C)CACAC(C/T)TTAG
(SEQ ID NO:68) and
5 GC(/T)TCAATG(/A)TGG(/A)GCAATG(/T)ACTGGA(/C)GTA(/G)ATTCCCA(/G)A
CATC (SEQ ID NO:73) ,
- 2) GGCATT(/C)TATGGAGTTGATTCTG(/A)TCCATT(/C)CACACC(/T)
TTAG (SEQ ID NO:68) and
CCATCAC(/T)ATAGAGGTTAC(/A)TG(/A)TCTGGCATT(/C)GC (SEQ ID NO:71),
- 10 3) GAAAGTGGG(/A/T)GGG(/A/T)A(/G)A(/C)T(/G)TAT(/C)AAAGTA(/G)
AATTCT(/G)CAAGAT(/C)TTA(/G)AAA(/G)G (SEQ ID NO:70) and
T(/G)CATG(/A)CTA(/G)AAC(/T)TCT(/A)ATC(/T)AAG(/A)GCATAACGACTATC(/
T) (SEQ ID NO:72), and
- 15 4) primer pairs that amplify the same region, or diagnostic portion thereof, from
the genome of a strain of *S. pneumoniae* as the primers provided in 1) to 3).

In an alternate embodiment, the nucleotide sequence analysis step comprises determining whether a polynucleotide obtained from *S. pneumoniae* selectively hybridises to a polynucleotide probe comprising one or more polymorphic regions of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, wherein such polymorphic regions are shown in Figure 2. More preferably, the nucleotide sequence analysis step comprises a plurality of said polynucleotide probes. In a particularly preferred embodiment, where hybridisation to a plurality of probes is used as a means of analysis, the plurality of polynucleotide probes are present as a microarray.

25 It has been noted that the method of analysing at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene does not enable the identification of all known *S. pneumoniae* serotypes, for example shared sequences were noted in the following cases; 6A and 6B; 10A and 17A, 10A and 23F, 23F and 23A; 15B, 15C, 22F and 22A; 17F, 35B, 35C and 42. Accordingly, in these
30 instances further analysis will need to be performed to determine the correct serotype. To this end, the present inventors have discovered that polymorphisms in the *wzy* and/or *wzx* genes can also be useful for *S. pneumoniae* serotyping.

Accordingly, in a further aspect the present invention provides a method of determining the serotype of *Streptococcus pneumoniae* in a sample, the method
35 comprising analysing at least a portion of the *wzy* and/or *wzx* gene(s).

In a preferred embodiment, the method comprises amplifying at least a portion of the *wzy* and/or *wzx* gene(s), and determining the length of the amplification product.

In a particularly preferred embodiment, at least a portion of the *wzy* and/or *wzx* gene(s) is amplified using primer pairs comprising a sequence selected from the group
5 consisting of:

- 1) GTAGGTGTAGTTTTTTCAGGGACTTTAATTTTATGCAGTG (SEQ ID NO:74) and
TCGCTTAACACAATGGCTTTAGAAGGTAGAG (SEQ ID NO:75),
- 2) GTTATTTTATTTTTTTTGTCTGGCATTGTATTCTTTATATCG (SEQ ID
10 NO:76) and CAAATTCATCGTTTGTATCCATTAACTGCATC (SEQ ID NO:77),
- 3) CTTATATCTAATTATGTTCCGCTCTATATTTATATGGGTTTGCTTTC
(SEQ ID NO:78) and TTTCTCTTCATTTTCCTGATAATTTTGTACTTCTGAATG
(SEQ ID NO:79),
- 4) ATGCTTTTAAATTTCTTATTCATATCTATTTTTC (SEQ ID NO:80) and
15 GTAAACAGAGAGCGAGTGATCATTTTAAACTTTTGG (SEQ ID NO:83),
- 5) G(/A)GATTTT(/G)TTTCAACCT(/C)GCAGTAATTTTAAACAA(/C)TC(/T)
G(/A) (SEQ ID NO:81) and
CCTGAAAACAA(/G)TACT(/C)ACTTTCTGAATTTTACAC(/T)GGA(/G)TATAAAG
(SEQ ID NO:82),
- 20 6) GTTTTATTGACTTTAAAGATGTTAGTTTCTTCGATTCCAG (SEQ ID
NO:84) and TTTTATTACTCTTCTTAAATCATAATGAATCGTACCAATCAAC
(SEQ ID NO:85),
- 7) GGATCAATGGCAACTATATTTACCCTACTCTCCACAG (SEQ ID
NO:86) and GAGTCGAAACCAACCGGAAAAAGCAATTGAG (SEQ ID NO:87),
- 25 8) CCTTTGGTTTATTATCCTACTTCCAAAACAGTTTATGC (SEQ ID
NO:88) and CATATATCTCTTTATCCTGTCAATATTGATTGGCATTTC (SEQ ID
NO:89),
- 9) GATATTAGCTATACCAACAATTGTTCTTTTCCTGTACTCAGTC (SEQ
ID NO:91) and GCATTTCTAGTACCGAACCATTGAAACTATCATCTG (SEQ ID
30 NO:93),
- 10) GAAATTATAGTCGGAGCTTTCATTTATATTAGTTTACTGGTTCTG
(SEQ ID NO:90) and CAGAATAAAGAGAGCTGTAATAGGTGCAACTTCATGC
(SEQ ID NO:93),
- 11) CTGTAATGTTTCTAATTAGTTCAGTATTTGCACTGGTTAATTC
35 (SEQ ID NO:94) and

- CCCGTATATCCATTACTAAGAACAAGGTTGTATATTCCTTC (SEQ ID NO:95),
- 12) GTTCTCATTAGTTCTGTATTTGCCCTTATTAATGTGC (SEQ ID NO:96) and CCATGGCTAAGTGCAAGATTATGAATCTCTCTC (SEQ ID NO:97),
- 5 13) GTTCTTATGTTTACCCTCAGCTTATATTGGCACAG (SEQ ID NO:98) and GATACCACAAATCTCCGAATTCTCTTAAAATAGATGG (SEQ ID NO:99),
- 14) TTAAGTAGTTCACAAGTGATAGTGAAGTTGGGATTGTC (SEQ ID NO:100) and CACTGAGATTATTTATTAGCTTTATCGGTAAGGTGGATAAG
- 10 (SEQ ID NO:101),
- 15) ATTACTTGTAATACTATGTATTCAACTAGTCA(/C)AGGATTTGAT GG (SEQ ID NO:103) and GAACAAATTTCCGTATCAGATTTGCGATTTTC (SEQ ID NO:104),
- 16) CCAATGAAAAGGAAAGTTCAATGTGTTTTGTTTCTGC (SEQ ID NO:102) and GGTGCTTCAGCAAAAATCCCCGTATTTCTTATCAG (SEQ ID NO:105),
- 17) TAGCTGATGTTCCGATAAATTATGGTGGGGTAATAATAG (SEQ ID NO:106) and CTGCGACACTGTATATACCTACATTATAACTACTAGACATTTGC (SEQ ID NO:107),
- 20 18) GCAACTTTGGTTCTAAAATTTTAGTCTTTTTAATGGTTCC (SEQ ID NO:108) and TGTAAACCCCAATATAGAAATTGTATTGAGAATAGCAGC (SEQ ID NO:109),
- 19) CGTTAATAGCTTATGTTCAACTGGTGATTGATTTTGG (SEQ ID NO:110) and TGATAGTTTTAGAAATAATATAAGGAATTGCAACTGCATGC
- 25 (SEQ ID NO:111),
- 20) TTCATGTC(/T)T(/C)TTTTG(/A)TCTAATCTGATTACAATTG(/C) TC(/T)A CAT CG(/A) (SEQ ID NO:113) and T(/C)GCATTTG(/T)GATCTGTCACAA(/G)TCAATAAGTTAAAACC (SEQ ID NO:114),
- 30 21) GGTAGGTATTTTAATTGGAGGAAGAGAGTCTTGAATGG (SEQ ID NO:112) and ATCTTCCCTTCATAAATTGACATAGGAAAAATAAGAGCC (SEQ ID NO:115),
- 22) CAATTCTAACTATGTCCAGTTTTATTTTCCACTCATCAG (SEQ ID NO:116) and GACGTGATAATAATAAGCTGCCATTCCTGTCTAAAACG (SEQ ID NO:117),
- 35

- 23) CGGCGGTATTAAGTAGAATATTAACACCTGAAGAGTATGGC (SEQ ID NO:118) and GGCAATCAGACTCAATAAGTTCATCCGTTTAAAGTTC (SEQ ID NO:119),
- 24) GGTATTGCCTTTCCTTTGATAACTTCTCCTTATTTATCAC (SEQ ID NO:120) and TGAACCTGTAACTCGACACCCAAAAATATAAATAAATGAG (SEQ ID NO:121),
- 25) GAATCGGACAATAGCACAGGTACGAACAAG (SEQ ID NO:123) and GCCATGTAATCAACTGACCAAGCAGGGTACTC (SEQ ID NO:124),
- 26) CAAAGGAACGTTATCAGCAATTGTGTCAAATTTTCAG (SEQ ID NO:122) and AAGATTAGGGCGCACAAAGTTTACTTGTTTTAGC (SEQ ID NO:125),
- 27) GTTATTTCTTCAAATCTGCTCATAGTTTTAACCTCATCAC (SEQ ID NO:126) and TATCTTGCGTTTTTCATCCCTTACAGTTATTAGGTTCAAAG (SEQ ID NO:127),
- 28) TTCTTCAAATCTTTTGACAGTCTTGACCTCTTCCTTG (SEQ ID NO:128) and TATCGTGCAATTCGAATCTGTTACAGCTAATACATTTAAAC (SEQ ID NO:129),
- 29) GTCCTGACGCTATCAAATATCATTTTCCCATTAAATCAC (SEQ ID NO:130) and CCCACATGTGATCAATAGGAGTGAAAATTCTCTATTC (SEQ ID NO:131),
- 30) GCTTTGGCTAACTTTTCATCAAAGATTTTAATTTTTTTTGTTAG (SEQ ID NO:133) and CCAGAGATAGCTGTAACACCAATTTTATCAATTCCCTTAG (SEQ ID NO:134),
- 31) CCTTTGGCTAATTTCTTGGACGATAATGAATTTGTATATG (SEQ ID NO:132) and CCACAAACATTAGCAATAAAGAAACCTAACAATCCC (SEQ ID NO:135),
- 32) GATCATACTCCCTATCATTACGACTCCCTATGTAACG (SEQ ID NO:137) and CCAAGAAATATCCAAACCTTTTGACACTAACTTAATCC (SEQ ID NO:138),
- 33) GTTGTTTTAGCTCAAGGAGGGATAATGTTGGCTTCG (SEQ ID NO:136) and GCTGATTTTACAAATAGGAAAATAGAGATTGCACCAAC (SEQ ID NO:139), and
- 34) a primer comprising a sequence selected from any one of SEQ ID NO's 144 to 333, and

35) a primer that can be used to amplify the same region, or diagnostic portion thereof, from the genome of a strain of *S. pneumoniae* as a primer provided as any one of SEQ ID NO's 75 to 139 or 144 to 333.

Guidance regarding the serotypes these primer pairs target, and the length of
5 resulting amplification products, is provided in Tables 2, 3 and 7.

It has been noted that some of the above primer pairs formed non-serotype specific amplicons, for example; PCR targeting serotype 6B also amplified 6A; PCR targeting 18C amplified all serotypes in serogroup 18; PCR targeting *wzx* (but not *wzy*) of serotype 23F, amplified three serotype 23A strains; PCR targeting *wzx* and *wzy* of
10 serotypes 33/37 amplified a 33A isolate and that targeting *wzx* amplified a serotype 33B isolate. Accordingly, in these instances further analysis will need to be performed to determine the correct serotype. For instance, traditional serological typing can be performed.

Serotype 3 does not contain *wzy* and *wzx* genes. Accordingly, upon obtaining
15 results using the method of analysing at least a portion of the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, the presence of serotype 3 can be confirmed by analysing the *orf2 (wze)-cap3A-cap3B* region. Preferably, serotype 3 is identified by amplifying a portion of the *orf2 (wze)-cap3A-cap3B* region using primer pairs selected from the group consisting of:

20 1) GCACAAAAAAAAGTTTGATATTCCCCTTGACAATAG (SEQ ID NO:140) and GCAGGATCTAAGGAGGCTTCAAGATTCAACTC (SEQ ID NO:141),

2) CGAACCTACTATTGAGTGTGATACTTTTATGGGATACAGAG (SEQ ID NO:142) and CTGACAGCATGAAAATATATAACCGCCCAACGAATAAG
25 (SEQ ID NO:143), and

3) primer pairs that amplify the same region, or diagnostic portion thereof, from the genome of a strain of *S. pneumoniae* as the primers provided in 1) or 2).

During routine analysis of a sample comprising bacteria it will typically be desirable to ensure that the sample being analysed actually contains *Streptococcus*
30 *pneumoniae*. Thus, it is preferred that the methods of the present invention include detecting any serotype of *Streptococcus pneumoniae* in the sample.

Such methods are known in the art and include, but are not limited to, amplifying portions of the *psaA* and/or pneumolysin genes followed by detection of the amplification products.

35 In a preferred embodiment, a portion of the *psaA* gene is amplified using primers comprising the sequence

TACATTACTCGTTCTCTTTCTTTCTGCAATCATTCTTG (SEQ ID NO:64) and TAGTAGCTGTCGCCTTCTTTACCTTGTTCTGC (SEQ ID NO:65), or primer pairs that amplify the same region, or diagnostic portion thereof, from the genome of a strain of *S. pneumoniae* as SEQ ID NO:64 and SEQ ID NO:65. In another preferred
 5 embodiment, a portion of the pneumolysin gene is amplified using primers comprising the sequence AGAATAATCCCACTCTTCTTGCGGTTGA (SEQ ID NO:66) and CATGCTGTGAGCCGTTATTTTTTCATACTG (SEQ ID NO:67) or primer pairs that amplify the same region, or diagnostic portion thereof, from the genome of a strain of *S. pneumoniae* as SEQ ID NO:66 and SEQ ID NO:67.

10 The present inventors have observed a strong correlation between the molecular capsular sequence typing techniques of the invention and the actual serotype of a strain as determined by traditional antibody based serological typing. However, the typing methods of the invention may be assisted by further serotyping the *S. pneumoniae* strain. For instance, to ensure recombination events have not occurred, upon typing
 15 with the methods of the invention the serotype can be confirmed by serologically typing for the strain suggested by the methods of the invention. Furthermore, the inventors have noted that a few serotypes are difficult to resolve using the methods of the invention, for example; 6A and 6B; 15B and 15C; 22F and 22A; and 35C and 42. Upon identification of any of these serotypes by the molecular techniques of the
 20 invention the serotype can be unequivocally typed using traditional serological methods.

In another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from those provided as SEQ ID NO's 2 to 63, or a fragment thereof which is at least 10 nucleotides in length, with the proviso
 25 that the polynucleotide does not comprise the entire *wzy* and/or *wzx* gene(s) of a *S. pneumoniae* serotype selected from the group consisting of: 1, 2, 4, 6A, 6B, 8, 9V, 14, 18C, 19F, 19A, 19B, 23F, 33F and 37, or the entire *wzx* gene of *S. pneumoniae* serotype 19C.

In a further aspect, the present invention provides an isolated polynucleotide
 30 comprising a sequence of nucleotides selected from the group consisting of: 1-AF532632, 10A-AF532633, 10A-AF532634, 10B-AY508586, 10F-AF532635, 10F-AF532636, 10F-AY508587, 11A-AF532637, 11A-AF532638, 11B-AF532639, 11C-AY508588, 11C-AY508589, 12A-AY508590, 12A-AY508591, 12F-AF532640, 12F-AF532641, 13-AF532642, 14-AF532643, 14-AF532644, 14-AF532645, 15A-
 35 AF532646, 15A-AF532647, 15B-AF532648, 15B-AF532649, 15B-AF532650, 15C-AF532651, 15C-AF532652, 15C-AY330714, 15C-AY330715, 15C-AY508592, 15C-

AY508593, 15F-AY508594, 15F-AY508595, 16A-AY508596, 16F-AF532653, 16F-AF532654, 17A-AF532655, 17A-AY508597, 17F-AF532656, 17F-AF532657, 18A-AF532658, 18A-AF532659, 18B-AF532660, 18C-AF532661, 18F-AF532662, 18F-AY330716, 18F-AY508598, 19A-AF532663, 19A-AF532664, 19B-AY508599, 19C-AY508600, 19C-AY508601, 19F-AF532665, 19F-AF532666, 19F-AF532667, 19F-AF532668, 2-AF532669, 20-AF532670, 21-AF532671, 21-AY508602, 22A-AF532672, 22F-AF532673, 23A-AF532674, 23A-AF532675, 23B-AF532676, 23B-AY330717, 23F-AF532677, 23F-AF532678, 23 F-AF532679, 24A-AY508603, 24B-AY508604, 24F-AY508605, 24F-AY508606, 24F-AY508607, 25F-AF532711, 27-AY508608, 28A-AY508609, 28F-AY508610, 28F-AY508611, 29-AF532680, 29-AY330718, 3-AF532681, 3-AF532682, 3-AF532683, 31-AF532684, 32A-AY508612, 32A-AY508613, 32F-AY508614, 33A-AF532685, 33B-AF532686, 33B-AY508615, 33C-AY508616, 33F-AF532687, 33F-AF532688, 33F-AF532689, 34-AF532690, 35A-AY508617, 35B-AF532691, 35C-AY508618, 35F-AF532692, 36-AY508619, 37-AF532713, 38-AF532712, 39-AY508620, 39-AY508621, 4-AF532693, 40-AY508622, 41A-AY508623, 41F-AY508624, 42-AY508625, 43-AY508626, 45-AY508628, 46-AY508629, 47A-AY508630, 47F-AY508631, 48-AY508632, 48-AY508633, 5-AF532696, 5-AF532697, 5-AY508634, 6A-AF532698, 6A-AF532699, 6A-AF532700, 6A-AF532701, 6A-AF532702, 6A-AY508641, 6B-AF532703, 6B-AF532704, 6B-AF532705, 7A-AY508635, 7B-AY508636, 7C-AF532706, 7F-AF532707, 8-AF532708, 9A-AY508637, 9L-AY508638, 9N-AF532709, 9V-AF532710 and 9V-AY508639 as provided in Figure 2, or a fragment thereof which is at least 10 nucleotides in length, with the proviso the polynucleotide does not comprise the 3' end of the *cpsA* gene to the 5' end of the *cpsB* gene of a *S. pneumoniae* serotype selected from the group consisting of: 1, 2, 3, 4, 6A, 6B, 8, 9V, 14, 18C, 19F, 19A, 23F, 33F and 37.

In a preferred embodiment, the polynucleotide of these aspects is at least 15 nucleotides, more preferably at least 20 nucleotides, more preferably at least 25 nucleotides, more preferably at least 30 nucleotides, more preferably at least 50 nucleotides in length, and even more preferably at least 100 nucleotides in length.

In a further aspect, the present invention provides an isolated polynucleotide consisting essentially of 10 to 50 contiguous nucleotides corresponding to a portion of the 3' end of the *cpsA* *S. pneumoniae* gene or the 5' end of the *cpsB* *S. pneumoniae* gene.

In a further aspect, the present invention provides a polynucleotide consisting essentially of 10 to 50 contiguous nucleotides corresponding to a portion of the *S. pneumoniae* *wzy* and/or *wzx* gene(s).

Preferably, said polynucleotide of 10 to 50 contiguous nucleotides comprises
5 one or more nucleotides which differ between different *S. pneumoniae* serotypes.

Polynucleotides of 10 to 50 contiguous nucleotides can be used as amplification primers, or as probes, for the identification of different *S. pneumoniae* serotypes.

Preferably the nucleotides which differ between *S. pneumoniae* serotypes correspond to one or more of positions as shown in Figure 2.

10 Preferably, the polynucleotide is detectably labelled. The label can be any suitable label known in the art including, but not limited to, radionuclides, enzymes, fluorescent, and chemiluminescent labels.

Also provided is a vector comprising a polynucleotide of the invention. Preferably, the vector is an expression vector. Furthermore, provided is a host cell
15 comprising a vector of the invention. Suitable vectors and host cells would be well known to those skilled in the art.

In yet another aspect, the present invention provides a composition comprising a plurality of polynucleotides according to the invention and an acceptable carrier or excipient. Preferably, the carrier or excipient is water or a suitable buffer. The
20 composition may be used in methods of typing different *S. pneumoniae* serotypes.

In a further aspect the present invention provides a microarray comprising a plurality of polynucleotides according to the invention. The microarray may be used in methods of typing different *S. pneumoniae* serotypes.

In another aspect, the present invention provides a kit comprising at least one
25 polynucleotide of the present invention.

Preferably, the polynucleotide is 10 to 50 nucleotides in length. In one embodiment, the kit further comprises reagents necessary for nucleic acid amplification. In another embodiment, the polynucleotide is detectably labelled and the kit further comprises means for detecting the labelled polynucleotide.

30 As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of
35 any other element, integer or step, or group of elements, integers or steps.

The invention is hereinafter described by way of the following non-limiting examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

5 Figure 1. The genomic sequence of *cpsA* (*wzg*) and *cpsB* (*wzh*) genes of serotype 4 of *S. pneumoniae* as published by Jiang et al. (2001) and deposited as GenBank Accession Number AF316639. The remaining 3' sequence of GenBank Accession Number AF316639 has not been provided. Nucleotides 1520 to 2965 encode *cpsA* whilst nucleotides 2967 to 3698 encode *cpsB*.

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Figure 2. Multiple sequence alignments for the region between the 3'-end of *cpsA* (*wzg*) and the 5'-end of *cpsB* (*wzh*) of 132 molecular capsular sequence types of *S. pneumoniae*. The alignment numbering start point "1" refer to the position "2470" of *S. pneumoniae* serotype 4 *cpsA* (*wzg*) gene (GenBank accession number: AF316639)

15 (Figure 1).

Figure 3. Phylogenetic tree inferred from sequences in the region between the 3'-end of *cpsA* (*wzg*) and the 5'-end of *cpsB* (*wzh*) genes for 132 molecular capsular sequence types of *S. pneumoniae*. Most of the tree input sequences are from Figure 2; for
20 GenBank accession numbers see Tables 1 and 8.

Figure 4. Phylogenetic tree of *wzx* genes of 83 *S. pneumoniae cps* serotypes. The tree is generated by the neighbour-joining method based on all nucleotide sites.

25 Figure 5. Phylogenetic tree of *wzy* genes of total 83 *S. pneumoniae cps* serotypes. The tree is generated by the neighbour-joining method based on all nucleotide sites.

Figure 6. Schematic representation of the closely related *wzx* genes. Each block represents *wzx* genes from one or more *S. pneumoniae* serotype *cps* gene cluster.
30 Similar patterns and shading represent regions with DNA sequence identity > 75% among different nucleotide sequences.

KEY TO THE SEQUENCE LISTING

SEQ ID NO:1 - Genomic sequence of *cpsA* (*wzg*) and *cpsB* (*wzh*) genes of serotype 4
35 of *S. pneumoniae* (Figure 1).

SEQ ID NO:2 - Partial sequence of strain 00-251-3185 *wzx* gene.

- SEQ ID NO:3 - Partial sequence of strain 01-122-0226 *wzx* gene.
SEQ ID NO:4 - Partial sequence of strain 01-192-2471 *wzx* gene.
SEQ ID NO:5 - Partial sequence of strain MA055100 *wzx* gene.
SEQ ID NO:6 - Partial sequence of strain NZSPN01/329 *wzx* gene.
5 SEQ ID NO:7 - Partial sequence of strain 00-256-1986 *wzx* gene.
SEQ ID NO:8 - Partial sequence of strain NZSPN01/276 *wzx* gene.
SEQ ID NO:9 - Partial sequence of strain 00-201-1422 *wzx* gene.
SEQ ID NO:10 - Partial sequence of strain 00-211-1669 *wzx* gene.
SEQ ID NO:11 - Partial sequence of strain 00S002 *wzx* gene.
10 SEQ ID NO:12 - Partial sequence of strain 00-251-3185 *wzy* gene.
SEQ ID NO:13 - Partial sequence of strain 01-122-0226 *wzy* gene.
SEQ ID NO:14 - Partial sequence of strain 01-192-2471 *wzy* gene.
SEQ ID NO:15 - Partial sequence of strain MA055100 *wzy* gene.
SEQ ID NO:16 - Partial sequence of strain NZSPN01/329 *wzy* gene.
15 SEQ ID NO:17 - Partial sequence of strain 00-256-1986 *wzy* gene.
SEQ ID NO:18 - Partial sequence of strain NZSPN01/276 *wzy* gene.
SEQ ID NO:19 - Partial sequence of strain 00-201-1422 *wzy* gene.
SEQ ID NO:20 - Partial sequence of strain 00-211-1669 *wzy* gene.
SEQ ID NO:21 - Partial sequence of strain 00S002 *wzy* gene.
20 SEQ ID NO:22 - Partial sequence of strain NZSPN01/509 *cpsI* and *wzx* genes.
SEQ ID NO:23 - Partial sequence of strain MA050408 *cpsI* and *wzx* genes.
SEQ ID NO:24 - Partial sequence of strain MA052433 *cpsI* and *wzx* genes.
SEQ ID NO:25 - Partial sequence of strain 00S009 *cpsI* and *wzx* genes.
SEQ ID NO:26 - Partial sequence of strain 99-325-0373 *cpsI* and *wzx* genes.
25 SEQ ID NO:27 - Partial sequence of strain NZSPN00/454 *cpsI* and *wzx* genes.
SEQ ID NO:28 - Partial sequence of strain NZSPN00/484 *cpsI* and *wzx* genes.
SEQ ID NO:29 - Partial sequence of strain 00-081-2291 *wzy* and *wzx* genes.
SEQ ID NO:30 - Partial sequence of strain 00S168 *wzy* and *wzx* genes.
SEQ ID NO:31 - Partial sequence of strain 00-280-1493 *wzy* and *wzx* genes.
30 SEQ ID NO:32 - Partial sequence of strain MA063073 *wzy* and *wzx* genes.
SEQ ID NO:33 - Partial sequence of strain NZSPN00/410 *wzy* and *wzx* genes.
SEQ ID NO:34 - Partial sequence of strain NZSPN01/243 *wzy* and *wzx* genes.
SEQ ID NO:35 - Partial sequence of strain MA063087 *wzy* and *wzx* genes.
SEQ ID NO:36 - Partial sequence of strain MA063207 *wzy* and *wzx* genes.
35 SEQ ID NO:37 - Partial sequence of strain 01S333 *wzx* gene.
SEQ ID NO:38 - Partial sequence of strain MA050663 *wciW* and *wzx* genes.

- SEQ ID NO:39 - Partial sequence of strain 01S319 *wciW* and *wzx* genes.
SEQ ID NO:40 - Partial sequence of strain NZSPN00/353 *wciW* and *wzx* genes.
SEQ ID NO:41 - Partial sequence of strain MA062610 *wciW* and *wzx* genes.
SEQ ID NO:42 - Partial sequence of strain MA053392 *wciW* and *wzx* genes.
5 SEQ ID NO:43 - Partial sequence of strain NZSPN00/319 *wciW* and *wzx* genes.
SEQ ID NO:44 - Partial sequence of strain NZSPN01/278 *wciW* and *wzx* genes.
SEQ ID NO:45 - Partial sequence of strain 01S009 *wciW* and *wzx* genes.
SEQ ID NO:46 - Partial sequence of strain MA052628 *wciW* and *wzx* genes.
SEQ ID NO:47 - Partial sequence of strain 00-081-2291 *cpsJ* and *wzy* genes.
10 SEQ ID NO:48 - Partial sequence of strain 00-280-1493 *cpsJ* and *wzy* genes.
SEQ ID NO:49 - Partial sequence of strain NZSPN00/410 *cpsJ* and *wzy* genes.
SEQ ID NO:50 - Partial sequence of strain NZSPN01/243 *cpsJ* and *wzy* genes.
SEQ ID NO:51 - Partial sequence of strain MA063073 *cpsJ* and *wzy* genes.
SEQ ID NO:52 - Partial sequence of strain 00S168 *cpsJ* and *wzy* genes.
15 SEQ ID NO:53 - Partial sequence of strain MA063087 *cpsJ* and *wzy* genes.
SEQ ID NO:54 - Partial sequence of strain MA063207 *cpsJ* and *wzy* genes.
SEQ ID NO:55 - Partial sequence of strain 01S319 *wzx* and *wzy* genes.
SEQ ID NO:56 - Partial sequence of strain NZSPN00/353 *wzx* and *wzy* genes.
SEQ ID NO:57 - Partial sequence of strain MA062610 *wzx* and *wzy* genes.
20 SEQ ID NO:58 - Partial sequence of strain MA053392 *wzx* and *wzy* genes.
SEQ ID NO:59 - Partial sequence of strain NZSPN00/319 *wzx* and *wzy* genes.
SEQ ID NO:60 - Partial sequence of strain NZSPN01/278 *wzx* and *wzy* genes.
SEQ ID NO:61 - Partial sequence of strain MA050663 *wzx* and *wzy* genes.
SEQ ID NO:62 - Partial sequence of strain MA052628 *wzx* and *wzy* genes.
25 SEQ ID NO:63 - Partial sequence of strain 01S009 *wzx* and *wzy* genes.
SEQ ID NO's 64 to 143 - Oligonucleotide primers provided in Table 2.
SEQ ID NO's 144 to 333 - Oligonucleotide primers provided in Table 7.
SEQ ID NO:334* - Sequence of serotype 33C *wzx* gene.
SEQ ID NO:335* - Sequence of serotype 10B *wzx* gene.
30 SEQ ID NO:336* - Sequence of serotype 10C *wzx* gene.
SEQ ID NO:337* - Sequence of serotype 10F *wzx* gene.
SEQ ID NO:338* - Sequence of serotype 11A *wzx* gene.
SEQ ID NO:339* - Sequence of serotype 11D *wzx* gene.
SEQ ID NO:340* - Sequence of serotype 12A *wzx* gene.
35 SEQ ID NO:341* - Sequence of serotype 12B *wzx* gene.
SEQ ID NO:342* - Sequence of serotype 12F *wzx* gene.

- SEQ ID NO:343* - Sequence of serotype 13 *wzx* gene.
SEQ ID NO:344* - Sequence of serotype 14 *wzx* gene.
SEQ ID NO:345* - Sequence of serotype 15A *wzx* gene.
SEQ ID NO:346* - Sequence of serotype 15B *wzx* gene.
5 SEQ ID NO:347* - Sequence of serotype 15C *wzx* gene.
SEQ ID NO:348* - Sequence of serotype 15F *wzx* gene.
SEQ ID NO:349* - Sequence of serotype 16A *wzx* gene.
SEQ ID NO:350* - Sequence of serotype 16F *wzx* gene.
SEQ ID NO:351* - Sequence of serotype 17A *wzx* gene.
10 SEQ ID NO:352* - Sequence of serotype 17F *wzx* gene.
SEQ ID NO:353* - Sequence of serotype 18A *wzx* gene.
SEQ ID NO:354* - Sequence of serotype 18B *wzx* gene.
SEQ ID NO:355* - Sequence of serotype 18F *wzx* gene.
SEQ ID NO:356* - Sequence of serotype 20 *wzx* gene.
15 SEQ ID NO:357* - Sequence of serotype 22A *wzx* gene.
SEQ ID NO:358* - Sequence of serotype 22F *wzx* gene.
SEQ ID NO:359* - Sequence of serotype 23A *wzx* gene.
SEQ ID NO:360* - Sequence of serotype 23B *wzx* gene.
SEQ ID NO:361* - Sequence of serotype 24B *wzx* gene.
20 SEQ ID NO:362* - Sequence of serotype 25A *wzx* gene.
SEQ ID NO:363* - Sequence of serotype 25F *wzx* gene.
SEQ ID NO:364* - Sequence of serotype 27 *wzx* gene.
SEQ ID NO:365* - Sequence of serotype 28A *wzx* gene.
SEQ ID NO:366* - Sequence of serotype 28F *wzx* gene.
25 SEQ ID NO:367* - Sequence of serotype 29 *wzx* gene.
SEQ ID NO:368* - Sequence of serotype 31 *wzx* gene.
SEQ ID NO:369* - Sequence of serotype 32A *wzx* gene.
SEQ ID NO:370* - Sequence of serotype 32F *wzx* gene.
SEQ ID NO:371* - Sequence of serotype 33A *wzx* gene.
30 SEQ ID NO:372* - Sequence of serotype 33B *wzx* gene.
SEQ ID NO:373* - Sequence of serotype 10A *wzx* gene.
SEQ ID NO:374* - Sequence of serotype 9N *wzx* gene.
SEQ ID NO:375* - Sequence of serotype 34 *wzx* gene.
SEQ ID NO:376* - Sequence of serotype 35A *wzx* gene.
35 SEQ ID NO:377* - Sequence of serotype 35B *wzx* gene.
SEQ ID NO:378* - Sequence of serotype 35C *wzx* gene.

- SEQ ID NO:379* - Sequence of serotype 35F *wzx* gene.
SEQ ID NO:380* - Sequence of serotype 36 *wzx* gene.
SEQ ID NO:381* - Sequence of serotype 38 *wzx* gene.
SEQ ID NO:382* - Sequence of serotype 39 *wzx* gene.
5 SEQ ID NO:383* - Sequence of serotype 40 *wzx* gene.
SEQ ID NO:384* - Sequence of serotype 41A *wzx* gene.
SEQ ID NO:385* - Sequence of serotype 41F *wzx* gene.
SEQ ID NO:386* - Sequence of serotype 42 *wzx* gene.
SEQ ID NO:387* - Sequence of serotype 43 *wzx* gene.
10 SEQ ID NO:388* - Sequence of serotype 44 *wzx* gene.
SEQ ID NO:389* - Sequence of serotype 45 *wzx* gene.
SEQ ID NO:390* - Sequence of serotype 46 *wzx* gene.
SEQ ID NO:391* - Sequence of serotype 47A *wzx* gene.
SEQ ID NO:392* - Sequence of serotype 47F *wzx* gene.
15 SEQ ID NO:393* - Sequence of serotype 48 *wzx* gene.
SEQ ID NO:394* - Sequence of serotype 48(1) *wzx* gene.
SEQ ID NO:395* - Sequence of serotype 7A *wzx* gene.
SEQ ID NO:396* - Sequence of serotype 7C *wzx* gene.
SEQ ID NO:397* - Sequence of serotype 7F *wzx* gene.
20 SEQ ID NO:398* - Sequence of serotype 9A *wzx* gene.
SEQ ID NO:399* - Sequence of serotype 9L *wzx* gene.
SEQ ID NO:400* - Sequence of serotype 33D *wzx* gene.
SEQ ID NO:401* - Sequence of serotype 33B *wzy* gene.
SEQ ID NO:402* - Sequence of serotype 10B *wzy* gene.
25 SEQ ID NO:403* - Sequence of serotype 10C *wzy* gene.
SEQ ID NO:404* - Sequence of serotype 10F *wzy* gene.
SEQ ID NO:405* - Sequence of serotype 11A *wzy* gene.
SEQ ID NO:406* - Sequence of serotype 11D *wzy* gene.
SEQ ID NO:407* - Sequence of serotype 12A *wzy* gene.
30 SEQ ID NO:408* - Sequence of serotype 12B *wzy* gene.
SEQ ID NO:409* - Sequence of serotype 12F *wzy* gene.
SEQ ID NO:410* - Sequence of serotype 13 *wzy* gene.
SEQ ID NO:411* - Sequence of serotype 14 *wzy* gene.
SEQ ID NO:412* - Sequence of serotype 15A *wzy* gene.
35 SEQ ID NO:413* - Sequence of serotype 15B *wzy* gene.
SEQ ID NO:414* - Sequence of serotype 15C *wzy* gene.

- SEQ ID NO:415* - Sequence of serotype 15F *wzy* gene.
SEQ ID NO:416* - Sequence of serotype 16A *wzy* gene.
SEQ ID NO:417* - Sequence of serotype 16F *wzy* gene.
SEQ ID NO:418* - Sequence of serotype 17A *wzy* gene.
5 SEQ ID NO:419* - Sequence of serotype 17F *wzy* gene.
SEQ ID NO:420* - Sequence of serotype 18A *wzy* gene.
SEQ ID NO:421* - Sequence of serotype 18B *wzy* gene.
SEQ ID NO:422* - Sequence of serotype 18F *wzy* gene.
SEQ ID NO:423* - Sequence of serotype 19C *wzy* gene.
10 SEQ ID NO:424* - Sequence of serotype 20 *wzy* gene.
SEQ ID NO:425* - Sequence of serotype 22A *wzy* gene.
SEQ ID NO:426* - Sequence of serotype 22F *wzy* gene.
SEQ ID NO:427* - Sequence of serotype 23A *wzy* gene.
SEQ ID NO:428* - Sequence of serotype 23B *wzy* gene.
15 SEQ ID NO:429* - Sequence of serotype 24B *wzy* gene.
SEQ ID NO:430* - Sequence of serotype 25A *wzy* gene.
SEQ ID NO:431* - Sequence of serotype 25F *wzy* gene.
SEQ ID NO:432* - Sequence of serotype 27 *wzy* gene.
SEQ ID NO:433* - Sequence of serotype 28A *wzy* gene.
20 SEQ ID NO:434* - Sequence of serotype 28F *wzy* gene.
SEQ ID NO:435* - Sequence of serotype 29 *wzy* gene.
SEQ ID NO:436* - Sequence of serotype 31 *wzy* gene.
SEQ ID NO:437* - Sequence of serotype 32A *wzy* gene.
SEQ ID NO:438* - Sequence of serotype 32F *wzy* gene.
25 SEQ ID NO:439* - Sequence of serotype 33A *wzy* gene.
SEQ ID NO:440* - Sequence of serotype 10A *wzy* gene.
SEQ ID NO:441* - Sequence of serotype 9N *wzy* gene.
SEQ ID NO:442* - Sequence of serotype 33D *wzy* gene.
SEQ ID NO:443* - Sequence of serotype 34 *wzy* gene.
30 SEQ ID NO:444* - Sequence of serotype 35A *wzy* gene.
SEQ ID NO:445* - Sequence of serotype 35B *wzy* gene.
SEQ ID NO:446* - Sequence of serotype 35C *wzy* gene.
SEQ ID NO:447* - Sequence of serotype 35F *wzy* gene.
SEQ ID NO:448* - Sequence of serotype 36 *wzy* gene.
35 SEQ ID NO:449* - Sequence of serotype 38 *wzy* gene.
SEQ ID NO:450* - Sequence of serotype 39 *wzy* gene.

- SEQ ID NO:451* - Sequence of serotype 40 *wzy* gene.
SEQ ID NO:452* - Sequence of serotype 41A *wzy* gene.
SEQ ID NO:453* - Sequence of serotype 41F *wzy* gene.
SEQ ID NO:454* - Sequence of serotype 42 *wzy* gene.
5 SEQ ID NO:455* - Sequence of serotype 43 *wzy* gene.
SEQ ID NO:456* - Sequence of serotype 44 *wzy* gene.
SEQ ID NO:457* - Sequence of serotype 45 *wzy* gene.
SEQ ID NO:458* - Sequence of serotype 46 *wzy* gene.
SEQ ID NO:459* - Sequence of serotype 47A *wzy* gene.
10 SEQ ID NO:460* - Sequence of serotype 47F *wzy* gene.
SEQ ID NO:461* - Sequence of serotype 48 *wzy* gene.
SEQ ID NO:462* - Sequence of serotype 48(1) *wzy* gene.
SEQ ID NO:463* - Sequence of serotype 7A *wzy* gene.
SEQ ID NO:464* - Sequence of serotype 7C *wzy* gene.
15 SEQ ID NO:465* - Sequence of serotype 7F *wzy* gene.
SEQ ID NO:466* - Sequence of serotype 9A *wzy* gene.
SEQ ID NO:467* - Sequence of serotype 9L *wzy* gene.
SEQ ID NO:468* - Sequence of serotype 33C *wzy* gene.
SEQ ID NO:469 - Sequence of serotype 9V *wzx* gene (Genbank accession no.
20 AF402095).
SEQ ID NO:470 - Sequence of serotype 19B *wzx* gene (Genbank accession no.
AF004325).
SEQ ID NO:471 - Sequence of serotype 19C *wzx* gene (Genbank accession no.
AF105116).
25 SEQ ID NO:472 - Sequence of serotype 19F *wzx* gene (Genbank accession no. U09239).
SEQ ID NO:473 - Sequence of serotype 2 *wzx* gene (Genbank accession no. AF026471).
SEQ ID NO:474 - Sequence of serotype 23F *wzx* gene (Genbank accession no.
AF057294).
SEQ ID NO:475 - Sequence of serotype 33F *wzx* gene (Genbank accession no.
30 AFAJ006986).
SEQ ID NO:476 - Sequence of serotype 37 *wzx* gene (Genbank accession no.
AJ131984).
SEQ ID NO:477 - Sequence of serotype 6A *wzx* gene (Genbank accession
no. AY078347).
35 SEQ ID NO:478 - Sequence of serotype 6B *wzx* gene (Genbank accession no.
AF316640).

- SEQ ID NO:479 - Sequence of serotype 8 *wzx* gene (Genbank accession no. AF316641).
SEQ ID NO:480 - Sequence of serotype 18C *wzx* gene (Genbank accession no. AF316642).
SEQ ID NO:481 - Sequence of serotype 9V *wzy* gene (Genbank accession no. AF402095).
5 SEQ ID NO:482 - Sequence of serotype 19B *wzy* gene (Genbank accession no. AF004325).
SEQ ID NO:483 - Sequence of serotype 19F *wzy* gene (Genbank accession no. U09239).
SEQ ID NO:484 - Sequence of serotype 2 *wzy* gene (Genbank accession no. AF026471).
10 SEQ ID NO:485 - Sequence of serotype 23F *wzy* gene (Genbank accession no. AF057294).
SEQ ID NO:486 - Sequence of serotype 33F *wzy* gene (Genbank accession no. AF006986).
SEQ ID NO:487 - Sequence of serotype 37 *wzy* gene (Genbank accession no. AJ131984).
15 SEQ ID NO:488 - Sequence of serotype 6A *wzy* gene (Genbank accession no. AY078347).
SEQ ID NO:489 - Sequence of serotype 6B *wzy* gene (Genbank accession no. AF316640).
20 SEQ ID NO:490 - Sequence of serotype 8 *wzy* gene (Genbank accession no. AF316641).
SEQ ID NO:491 - Sequence of serotype 18C *wzy* gene (Genbank accession no. AF316642).
SEQ ID NO:492 - Consensus sequence for 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene of *S. pneumoniae* strains that were analysed.

25

* Indicates that these sequences were extracted from unannotated sequence data from the Sanger Institute website.

DETAILED DESCRIPTION OF THE INVENTION

30 Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry).

35

As used herein, the term "nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene" at least refers to the region spanning from

nucleotide 2470 to nucleotide 3268 of Figure 1. Figure 1 provides the genomic sequence of *cpsA* (*wzg*) and *cpsB* (*wzh*) genes of serotype 4 as published by Jiang et al. (2001) and submitted as GenBank Accession Number AF316639. As the skilled addressee would be aware, the same region from other serotypes of *S. pneumoniae* can be identified using standard techniques such as DNA cloning, sequencing and nucleotide sequence alignment. Such techniques are described in further detail in the Examples section. In addition, these techniques have been used to determine the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene from many different serotypes of *S. pneumoniae*, the results of which, including a consensus sequence for this region, are also provided in Figure 2.

As used herein, the term "primer pairs that amplify the same region, or diagnostic portion thereof, from the genome of a strain of *S. pneumoniae*", or variations thereof, refers to the capability of the skilled addressee to determine where the identified primers of the claimed invention hybridize the *S. pneumoniae* genome of a particular strain(s), and subsequent ability to design alternate primers which can be used for the same purpose as the primers defined herein. Typically, these alternate primers will hybridize the same region of the genome but be larger or smaller in size, or these alternate primers will hybridize to a region of the genome which is in close proximity, for example within 500 basepairs, to where the specifically defined primers hybridize. Naturally, the term "diagnostic portion thereof" refers to the alternate primers being capable of amplifying a portion of the region of the defined primers but still capable of amplifying enough of the region to determine the serotype of a particular *S. pneumoniae* isolate.

25 General Techniques

Unless otherwise indicated, the recombinant DNA and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A

Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present), and are incorporated herein by reference.

5 Detection of Polymorphisms

Any technique known in the art can be used to detect a polymorphism described herein. Examples of such techniques include, but are not limited to, sequencing of the DNA at one or more of the relevant positions; differential hybridisation of an oligonucleotide probe designed to hybridise at the relevant positions of a particular *S. pneumoniae* serotype(s); denaturing gel electrophoresis following digestion with an appropriate restriction enzyme, preferably following amplification of the relevant DNA regions; S1 nuclease sequence analysis; non-denaturing gel electrophoresis, preferably following amplification of the relevant DNA regions; conventional RFLP (restriction fragment length polymorphism) assays; selective DNA amplification using oligonucleotides which are matched for a particular *S. pneumoniae* serotype(s) unmatched for other *S. pneumoniae* serotype(s); or the selective introduction of a restriction site using a PCR (or similar) primer matched for a particular *S. pneumoniae* serotype(s), followed by a restriction digest. As outlined above, it is preferred that the nucleotide sequence between the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene is characterized by DNA sequencing, whilst the analysis at least a portion of the *wzy* and/or *wzx* gene is performed by procedures involving the detection of amplification products.

In one embodiment, the informative serotyping information provided herein is adapted to produce a molecular capsular sequence typing database as generally described by Robertson et al. (2004).

PCR-based methods of detection may rely upon the use of primer pairs, at least one of which binds specifically to a region of interest in one or more, but not all, serotypes. Unless both primers bind, no PCR product will be obtained. Consequently, the presence or absence of a specific PCR product may be used to determine the presence of a sequence indicative of a specific *S. pneumoniae* serotype(s). However, as mentioned, only one primer need correspond to a region of heterogeneity in the genes/regions of interest. The other primer may bind to a conserved or heterogeneous region within said gene/region or even a region within another part of the *S. pneumoniae* genome, whether said region is conserved or heterogeneous between serotypes.

Alternatively, primers that bind to conserved regions of the *S. pneumoniae* genome but which flank a region whose length varies between serotypes may be used. In this case, a PCR product will always be obtained when *S. pneumoniae* bacteria are present but the size of the PCR product varies between serotypes. Examples of such
5 varying amplification product lengths are disclosed herein in relation to the *wzy* and *wzx* genes.

Furthermore, a combination of specific binding of one or both primers and variations in the length of PCR primer may be used as a means of identifying particular molecular serotypes.

10 In some cases, PCR and other specific hybridisation- based serotyping methods will involve the use of nucleotide primers/probes which bind specifically to a region of the genome of a *S. pneumoniae* serotype which includes a nucleotide which varies between two or more serotypes. Thus the primers/probes may comprise a sequence which is complementary to one of such regions. Where positions of heterogeneity are
15 close together (for instance within 5 or so nucleotides), it may be desirable to use a primer/probe which hybridises specifically to a region of the *S. pneumoniae* genome that comprises two or more positions of heterogeneity. Such primers/probes are likely to have improved specificity and reduce the likelihood of false positives.

PCR techniques that utilize fluorescent dyes may be used in the detection
20 methods of the present invention. These include, but are not limited to, the following five techniques.

i) Fluorescent dyes can be used to detect specific PCR amplified double stranded DNA product (e.g. ethidium bromide, or SYBR Green I).

25 ii) The 5' nuclease (TaqMan) assay can be used which utilizes a specially constructed primer whose fluorescence is quenched until it is released by the nuclease activity of the Taq DNA polymerase during extension of the PCR product.

iii) Assays based on Molecular Beacon technology can be used which rely on a specially constructed oligonucleotide that when self-hybridized quenches fluorescence (fluorescent dye and quencher molecule are adjacent). Upon hybridization to a specific
30 amplified PCR product, fluorescence is increased due to separation of the quencher from the fluorescent molecule.

iv) Assays based on Amplifluor (Intergen) technology can be used which utilize specially prepared primers, where again fluorescence is quenched due to self-hybridization. In this case, fluorescence is released during PCR amplification by
35 extension through the primer sequence, which results in the separation of fluorescent and quencher molecules.

v) Assays that rely on an increase in fluorescence resonance energy transfer can be used which utilize two specially designed adjacent primers, which have different fluorochromes on their ends. When these primers anneal to a specific PCR amplified product, the two fluorochromes are brought together. The excitation of one
5 fluorochrome results in an increase in fluorescence of the other fluorochrome.

Probes and primers may be fragments of DNA isolated from nature or may be synthetic. In one embodiment, primers/probes have a high melting temperature of $>70^{\circ}\text{C}$ so that they may be used in rapid cycle PCR. Preferably, the primers/probes comprise at least 10, 15 or 20 nucleotides. Typically, primers/probes consist of fewer
10 than 50 or 30 nucleotides. Primers/probes are generally polynucleotides comprising deoxynucleotides. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5'
15 ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Primers/probes may be labelled with any suitable detectable label such as radioactive atoms, fluorescent molecules or biotin.

The primers be synthesized using techniques which are well known in the art.
20 Generally, the primers can be made using synthesizing machines which are commercially available.

If required, in order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme sites appended to their 5' ends. Thus, all nucleotides of the primers are derived from the gene sequence of interest or sequences
25 adjacent to that gene except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art.

A sample to be typed for the presence and/or identification of a *S. pneumoniae* serotype may be from a bacterial culture or a clinical sample from a patient, typically a human patient. Clinical samples may be cultured to produce a bacterial culture.
30 However, it is also possible to test clinical samples directly with a culturing step.

The methods of the present invention can be used in a multi-step serotyping strategy. An example of such a multi-step serotyping strategy (algorithm) is shown in Table 6. However, a variety of other strategies are envisaged and can be designed by the skilled person using the sequence heterogeneity information presented herein. In
35 particular, it is preferred that the serotyping procedure comprise at least one analysis step based on analysing one or regions between the 3' end of the *cpsA* gene and the 5'

end of the *cpsB* gene. This analysis may optionally be combined with an analysis of one or more regions within the *wzy* and/or *wzx* genes.

Microarrays

5 Analysis of *S. pneumoniae* genomic sequences using the above techniques may take place in solution followed by standard resolution using methods such as gel electrophoresis. However in a preferred aspect of the invention, the primers/probes are immobilised onto a solid substrate to form arrays.

10 The polynucleotide probes are typically immobilised onto or in discrete regions of a solid substrate. The substrate may be porous to allow immobilisation within the substrate or substantially non-porous, in which case the probes are typically immobilised on the surface of the substrate. Examples of suitable solid substrates include flat glass (such as borosilicate glass), silicon wafers, mica, ceramics and organic polymers such as plastics, including polystyrene and polymethacrylate. It may
15 also be possible to use semi-permeable membranes such as nitrocellulose or nylon membranes, which are widely available. The semi-permeable membranes may be mounted on a more robust solid surface such as glass. The surfaces may optionally be coated with a layer of metal, such as gold, platinum or other transition metal.

20 Preferably, the solid substrate is generally a material having a rigid or semi-rigid surface. In preferred embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, raised regions or etched trenches. It is also preferred that the solid substrate is suitable for the high density application of DNA sequences in discrete areas of typically from 50 to 100 μm ,
25 giving a density of 10000 to 40000 cm^{-2} .

30 The solid substrate is conveniently divided up into sections. This may be achieved by techniques such as photoetching, or by the application of hydrophobic inks, for example teflon-based inks (Cel-line, USA). Discrete positions, in which each different probes are located may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc.

35 Attachment of the library sequences to the substrate may be by covalent or non-covalent means. The library sequences may be attached to the substrate via a layer of molecules to which the library sequences bind. For example, the probes may be labelled with biotin and the substrate coated with avidin and/or streptavidin. A convenient feature of using biotinylated probes is that the efficiency of coupling to the solid substrate can be determined easily. Since the polynucleotide probes may bind

only poorly to some solid substrates, it is often necessary to provide a chemical interface between the solid substrate (such as in the case of glass) and the probes. Thus, the surface of the substrate may be prepared by, for example, coating with a chemical that increases or decreases the hydrophobicity or coating with a chemical that allows covalent linkage of the polynucleotide probes. Some chemical coatings may both alter the hydrophobicity and allow covalent linkage. Hydrophobicity on a solid substrate may readily be increased by silane treatment or other treatments known in the art. Examples of suitable chemical coatings include polylysine and poly(ethyleneimine). Further details of methods for the attachment of are provided in US 6,248,521.

Techniques for producing immobilised arrays of nucleic acid molecules have been described in the art. A useful review is provided in Schena *et al.* (1998), which also gives references for the techniques described therein.

Microarray-manufacturing technologies fall into two main categories—synthesis and delivery. In the synthesis approaches, microarrays are prepared in a stepwise fashion by the *in situ* synthesis of nucleic acids from biochemical building blocks. With each round of synthesis, nucleotides are added to growing chains until the desired length is achieved. A number of prior art methods describe how to synthesise single-stranded nucleic acid molecule libraries *in situ*, using for example masking techniques (photolithography) to build up various permutations of sequences at the various discrete positions on the solid substrate. US 5,837,832 describes an improved method for producing DNA arrays immobilised to silicon substrates based on very large scale integration technology. In particular, U.S. Patent No. 5,837,832 describes a strategy called "tiling" to synthesize specific sets of probes at spatially-defined locations on a substrate which may be used to produced the immobilised DNA libraries of the present invention. US 5,837,832 also provides references for earlier techniques that may also be used.

The delivery technologies, by contrast, use the exogenous deposition of prepared biochemical substances for chip fabrication. For example, DNA may also be printed directly onto the substrate using for example robotic devices equipped with either pins (mechanical microspotting) or piezo electric devices (ink jetting). In mechanical microspotting, a biochemical sample is loaded into a spotting pin by capillary action, and a small volume is transferred to a solid surface by physical contact between the pin and the solid substrate. After the first spotting cycle, the pin is washed and a second sample is loaded and deposited to an adjacent address. Robotic control systems and multiplexed printheads allow automated microarray fabrication. Ink jetting

involves loading a biochemical sample, such as a polynucleotide into a miniature nozzle equipped with a piezoelectric fitting and an electrical current is used to expel a precise amount of liquid from the jet onto the substrate. After the first jetting step, the jet is washed and a second sample is loaded and deposited to an adjacent address. A
5 repeated series of cycles with multiple jets enables rapid microarray production.

In one embodiment, the microarray is a high density array, comprising greater than about 50, preferably greater than about 100 or 200 different nucleic acid probes. Such high density probes comprise a probe density of greater than about 50, preferably greater than about 500, more preferably greater than about 1,000, most preferably
10 greater than about 2,000 different nucleic acid probes per cm². The array may further comprise mismatch control probes and/or reference probes (such as positive controls).

Microarrays of the invention will typically comprise a plurality of primers/probes as described above. The primers/probes may be grouped on the array in any order.

15 Elements in an array may contain only one type of probe/primer or a number of different probes/primers.

Detection of binding of *S. pneumoniae* DNA to immobilised probes/primers may be performed using a number of techniques. For example, the immobilised probes which are specific for one or a number of serotypes, may function as capture probes.
20 Following binding of the genomic DNA to the array, the array is washed and incubated with one or more labelled detection probes which hybridise specifically to regions of the *S. pneumoniae* genome which are conserved (for example the *S. pneumoniae* *psaA* or pneumolysin probes/primers described herein could be utilized for this purpose). The binding of these detection probes may then be determined by detecting the
25 presence of the label. For example, the label may be a fluorescent label and the array may be placed in an X-Y reader under a charge-coupled device (CCD) camera.

Other techniques include labelling the genomic DNA prior to contact with the array (using nick-translation and labelled dNTPs for example). Binding of the genomic DNA can then be detected directly.

30 It is also possible to employ a single PCR amplification step using labelled dNTPs. In this embodiment, the genomic DNA fragment binds to a first primer present in the array. The addition of polymerase, dNTPs, including some labelled dNTPs and a second primer results in synthesis of a PCR product incorporating labelled nucleotides. The labelled PCR fragment captured on the plate may then be detected.

35 A number of available detection techniques do not require labels but instead rely on changes in mass upon ligand binding (e.g. surface plasmon resonance- SPR). The

principles of SPR and the types of solid substrates required for use in SPR (e.g. BIACore chips) are described in Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

Examples of the utilization of microarrays in genotyping include the use of
5 microarrays to differentiate between closely related *Cryptosporidium parvum* isolates and *Cryptosporidium* species (Straub *et al.*, 2002), the use of microarrays to differentiate between species of *Listeria* (Volokhov *et al.*, 2002), and the use of microarrays to differentiate within species of *Staphylococcus aureus* (van Leeuwen *et al.*, 2003). The detection principles applied in these studies can be used with the
10 polymorphisms/primers/probes identified by the present inventors to identify different serotypes of *S. pneumoniae* in a sample.

In the present instance, according to 800bp *cpsA-cpsB* alignment results (Figure 2) regions, such as the first 20 nucleotides provided in Figure 2, are scanned to see whether they contains polymorphisms. Where polymorphisms occur, probes can be
15 designed for each "type" (allele)-specific probes (and name them as 1-1, 1-2 , etc.), which will cover all the *cpsA-cpsB* regions for all the known sequence types. The combination of all the above allele-specific probes (about or less than 20 allele x 40~50 =800~1000 probes all together) hybridisation results will define the microarray hybridisation types like MLST (1-0-10-----etc), which would be nearly equal to the
20 sequencing results. Bioinformatics software will tell which sequence type the "specimen/strain" is.

Kits

In one embodiment, kits of the present invention include, in an amount
25 sufficient for at least one assay, a polynucleotide probe of the invention which preferentially hybridizes to a target nucleic acid sequence in a test sample under hybridization assay conditions. Kits containing multiple probes are also contemplated by the present invention where the multiple probes are designed to target different nucleic acid sequences from different *S. pneumoniae* serotypes and may include
30 distinct labels which permit the probes to be differentially detected in a test sample. Kits according to the present invention may further comprise at least one of the following: (i) one or more amplification primers for amplifying a target sequence contained in or derived from the target nucleic acid; (ii) a capture probe for isolating and purifying target nucleic acid present in a test sample; and (iii) if a capture probe is
35 included, a solid support material (e.g., magnetically responsive particles) for

immobilizing the capture probe, either directly or indirectly, in a test sample. Kits of the present invention may further include one or more helper probes.

Typically, the kits will also include instructions recorded in a tangible form (e.g., contained on paper or an electronic medium) for using the packaged polynucleotide in a detection assay for determining the presence or amount of a target nucleic acid sequence in a test sample. The assay described in the written instructions may include steps for isolating and purifying the target nucleic acid prior to detection with the polynucleotide probe, and/or amplifying a target sequence contained in the target nucleic acid. The instructions will typically indicate the reagents and/or concentrations of reagents and at least one assay method parameter which might be, for example, the relative amounts of reagents to use per amount of sample. In addition, such specifics as maintenance, time periods, temperature and buffer conditions may also be included.

15 Uses

As discussed above, *S. pneumoniae* is a leading cause of morbidity and mortality causing invasive disease such as meningitis and pneumonia as well as more localised disease such as acute otitis media and sinusitis. Continued surveillance is critical to monitor vaccine efficacy and changes in incidence and distribution of colonising and invasive serotypes. Any increase in disease caused by previously uncommon nonvaccine serotypes could necessitate a change in vaccine composition. Thus, the detection methods, probes/primer and microarrays of the invention may be used to monitor the epidemiology of invasive *S. pneumoniae* infections to assist in disease control and to inform vaccine policy.

25 The molecular typing methods of the invention may also assist in comprehensive serotype identification that will be useful for epidemiological and other related studies that will be needed to monitor *S. pneumoniae* before and after introduction of *S. pneumoniae* vaccines.

30

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EXAMPLES

EXAMPLE 1 - Serotyping based on the polymorphisms of the 3' end of the *cpsA* gene and the 5' end of the *cpsB* gene, combined in some instances with the analysis of the *wzx* and/or *wzy* genes

5 MATERIALS AND METHODS

Pneumococcal reference panels (Table 1)

Reference panels 1-4, which consisted of 118 isolates, were kindly provided and serotyped by colleagues in Australia and Canada. All had been serotyped using the standard Quellung method and included all 23 serotypes represented in the polysaccharide vaccine, and 28 additional serotypes; there were multiple isolates of 40 serotypes and five isolates that could not be serotyped with available antisera. Reference panel 5 consisted of 21 invasive isolates from our diagnostic laboratory at the Centre for Infectious Diseases and Microbiology (CIDM), Sydney, for which serotypes were known at the beginning of the study. These five reference panels were used for the development and preliminary evaluation of molecular capsular sequence methods. Panels 2 and 4 were tested by molecular capsular sequence, initially, without knowledge of the conventional serotyping (CS) results.

Clinical isolates

179 consecutive *S. pneumoniae* clinical isolates from normally sterile sites, collected during the period January 1999 to June 2001, by the CIDM diagnostic laboratory, were studied; 21 were randomly selected to make up reference panel 5 (see above). Dr Diana Martin, Institute of Environmental Science and Research (ESR), Wellington, New Zealand provided 103 clinical isolates from diagnostic laboratories throughout New Zealand. Clinical isolates were initially tested using the MCT method, without knowledge of their CS results (single-blind study). Isolates were retrieved from storage by subculture on blood agar plates (Columbia II agar base supplemented with 5% horse blood) and incubated overnight at 37°C CO₂ incubator.

30

35

Table 1. Conventional serotyping (CS) and molecular capsular typing (MCT) results of *S. pneumoniae* strains used in this study.

Strain numbers and geographic origin	CS ¹	MCT-Seq ²	MCT-PCR ²	GenBank ² accession numbers
Reference panel 1³				
Queensland				
00S001	19F	19F	19F	AF532666
00S002	6B	6B-q	6B	AF532705;
				AY163180, AY163190
00S006	19A	19A	19A	AF532663
00S009	23F	23F-g	23F	AF532677;
				AY163214, AY163232
00S014	1	1	1	AF532632
00S016	9V	9V	9V	AF532710
00S023	5	5-q		AF532697
00S033	17F	17F-35B		AF532657
00S036	11A	11A-q		AF532637
00S042	18C	18C/18B	18C	AF532661
00S059	9N	9N		AF532709
00S063	12F	12F		AF532640
00S067	8	8	8	AF532708
00S124	7F	7F		AF532707
00S154	15B	15B-q		AF532649
00S159	4	4	4	
00S168	33F	33F-q	33F/37	AF532687;
				AY163199, AY163221
00S246	22F	22F		AF532673
00S259	2	2-q	2	AF532669
00S300	22A	22A		AF532672
01S009	18C	18C/18B	18C	
01S020	7C	7C		AF532706
01S043	10A	10A-q		AF532633
01S143	3	3	3	AF532682
01S146	10F	10F		AF532635
01S305	20	20/13		AF532670
01S319	18A	18A	18C	AF532658;
				AY163208, AY163224
01S333	33B	33B	33F-X; 33F-Y-NEG	AF532686
01S358	35B	35B		AF532691
01S666	14	14-g	14	AF532643
01S682	16F	16F		AF532653
01S691	15C	15C-q		AF532651
01S753	4	4	4	AF532693
Reference panel 2⁴				
Victoria				
0013856	35B	35B		
0013976	6A	6A-ca	6B	
0017666	9V	9V	9V	
0019532	23F	23F-g	23F	
0102206	8	8	8	
0103678	19F	19F	19F	
0104603	6B	6B-q	6B	
0104604	22F	22F		

0104912	4	4	4	
0105015	14	14-g	14	AF532644
Reference panel 3⁵				
Canada				
MA007753	31	31		AF532684
MA007765	5	5-q		
MA008229	10F	10F		AF532636
MA008562	11A	11A-q		
MA008622	31	31		
MA050408	23A	23A-23F	23F-X; 23F-Y-NEG	AF532674
MA050663	18F	18F	18C	AF532662; AY163207, AY163230
MA050910	2	2-q	2	
MA050947	38	38/25F		AF532712
MA051117	22A	22A		
MA051617	35F	35F		AF532692
MA051950	31 (see Example 2)	31		AF532695
MA052002	15A	15A-ca1		AF532646
MA052150	11B	11B		AF532639
MA052217	7C	7C		
MA052253	17F	17F-35B		
MA052433	23A	23A-ca	23F-X; 23F-Y-NEG	AF532675
MA052434	15A	15A-ca2		AF532647
MA052628	18C	18C/18B	18C	-; AY163215, AY163231
MA052979	15C	15C-ca		AF532652
MA053096	20	20/13		
MA053188	15B	15B-q		
MA053392	18B	18B/18C	18C	AF532660; AY163211, AY163227
MA053567	12F	12F		
MA053684	38	38/25F		
MA053782	13	13/20		AF532642
MA053909	35B	35B		
MA054004	13	13/20		
MA054006	13	13/20		
MA054242	38	38/25F		
MA054294	16F	16F		
MA054338	35F	35F		
MA054357	1	1	1	
MA054490	34	34		AF532690
MA054545	3	3	3	
MA054735	10A	10A-q		
MA054832	34	34		
MA054883	7F	7F		
MA055006	9V	9V	9V	
MA055054	22F	22F		
MA055100	6A	6A-ca	6B	AF532702; AY163174, AY163184
MA056382	19A	19A	19A	AF532664
MA059287	25F	25F/38		AF532711
MA061296	41A (see Example 2)	41A		AF532694
MA061378	17A	17A		AF532655

MA061938	21	21		AF532671
MA062028	29	29		AF532680
MA062610	18B	18B/18C	18C	-;
				AY163210, AY163226
MA063013	9N	9N		
MA063073	33F	33F-g/33A	33F/37	AF532689;
				AY163201, AY163220
MA063087	33A	33A/33F-g	33F/37	AF532685;
				AY163204, AY163222
MA063189	Nonserotypeable	No-amplicon		
MA063207	37	37	33F/37	AF532713;
				AY163205, AY163223
MA063745	Nonserotypeable	Nonserotypeable-ca		AF532715
Reference panel 4⁶				
New South Wales				
00-177-0145	19A	19A	19A	
01-184-0091	18C	18C/18B	18C	
00-237-0230	17F	17F-35B		AF532656
01-273-0175	16F	16F		
00-201-0306	14	14-g	14	
01-117-0176	13	13/20		
01-239-0283	12F	12F		
00-206-0233	11A	11A-q		
00-222-0342	10A	10A-23F	23F-NEG	AF532634
01-180-0149	1	1	1	
01-122-0226	6A	6A-ca	6B	AF532698;
				AY163172, AY163182
99-308-0385	4	4		
00-234-0199	38	38/25F		
00-074-0065	35F	35F		
00-280-0121	3	3	3	
99-308-0290	23F	23F-g	23F	
00-244-0101	22F	22F		
00-250-0302	22A	22A		
00-244-0108	20	20/13		
01-009-0101	19F	19F	19F	AF532668
01-254-0150	7F	7F		
Reference panel 5⁷				
New South Wales,				
(CIDM)				
00-163-0650	14	14-g	14	
00-141-1399	19F	19F	19F	
00-070-0212	23F	23F-g	23F	
01-018-1842	4	4	4	
00-201-1422	6B	6B-g	6B	AF532703;
				AY163178, AY163188
00-180-2749	9V	9V	9V	
00-339-3084	9N	9N		
00-017-0985	11A	11A-q		
01-072-0391	12F	12F		AF532641
00-315-3100	15B	15B-c		AF532648
99-259-1456	18C	18C/18B	18C	
00-273-2862	4	4	4	
00-081-2291	33F	33F-g/33A	33F/37	-;
				AY163198, AY163216
00-118-2067	5	5-c		AF532696

01-175-0822	7F	7F		
00-324-0978	8	8	8	
00-152-1664	22F	22F		
00-211-1414	22F	22F		
00-200-0078	14	14-g	14	
00-118-0159	19F	19F	19F	
00-310-1104	4	4	4	
Clinical isolates				
New South Wales,				
(CIDM)⁸				
01-192-3558	6B	6B-g	6B	
01-192-2471	6A	6A-c	6B	AF532699; AY163173, AY163183
01-192-1205	6B	6B-g	6B	
01-191-1265	14	14-g	14	
01-189-0296	19F	19F	19F	
01-185-0511	15B	15B-22F		AF532650
01-184-0328	8	8	8	
01-179-2448	14	14-g	14	
01-178-0165	14	14-g	14	
01-176-3302	1	1	1	
01-173-2782	4	4	4	
01-170-0873	9V	9V	9V	
01-159-0505	14	14-g	14	
01-157-3399	4	4	4	
01-157-3394	4	4	4	
01-157-2062	4	4	4	
01-152-3295	14	14-g	14	
01-150-3706	14	14-g	14	
01-144-1862	7F	7F		
01-143-3353	4	4	4	
01-124-2300	12F	12F		
01-117-1910	4	4	4	
01-096-2050a	9V	9V	9V	
01-096-2050b	9V	9V	9V	
01-096-2027	9V	9V	9V	
01-077-1533	7F	7F		
01-075-3257	9N	9N		
01-058-3662	14	14-g	14	
01-048-1320	19A	19A	19A	
01-005-0764	19F	19F	19F	AF532650
00-361-1217	6B	6B-q	6B	
00-357-1164	14	14-g	14	
00-339-2918	9N	9N		
00-324-0977	8	8	8	
00-315-2993	23F	23F-g= 10A-23F	23F	
00-315-2254	23F	23F-g= 10A-23F	23F	
00-310-0630	14	14-g	14	
00-303-0303	19F	19F	19F	
00-293-1660	19F	19F	19F	
00-280-1493	33F	33F-q	33F/37	-;
00-267-0653	8	8	8	AY163200, AY163217

00-258-1120	14	14-g	14	
00-257-0881	9V	9V	9V	
00-256-1986	6A	6A-ca	6B	
00-251-3185	6A	6A-6B-g=	6B	-;
		6B-g		AY163176, AY163186
00-245-3950	23F	23F-g=	23F	AF532700;
		10A-23F		AY163171, AY163181
00-243-2229	3	3	3	
00-242-0394	14	14-g	14	
00-241-2964	9V	9V	9V	
00-238-3448	23F	23F-g=	23F	
		10A-23F		
00-235-3584	19F	19F	19F	AF532665
00-228-3777	35B	35B		
00-225-1482	3	3	3	
00-225-0333	19F	19F	19F	
00-217-3003	4	4	4	
00-211-1669	6B	6B-c	6B	AF532704;
				AY163179, AY163189
00-211-0475	22F	22F		
00-211-0469	22F	22F		
00-209-3409	3	3	3	
00-208-0179	4	4	4	
00-200-1013	14	14-g	14	
00-200-1012	14	14-g	14	
00-199-0498	4	4	4	
00-196-2923	9V	9V	9V	
00-192-2087	19A	19A	19A	
00-184-1203	6B	6B-q	6B	
00-181-1568	23F	23F-g=	23F	
		10A-23F		
00-181-1567	23F	23F-g=	23F	
		10A-23F		
00-173-3686	4	4	4	
00-164-1705	6B	6B-q	6B	
00-163-1533	14	14-g	14	
00-149-1265	7F	7F		
00-149-1264	7F	7F		
00-143-1473	15B	15B-22F		
00-138-3435	3	3	3	
00-118-2891	19F	19F	19F	
00-093-1315	3	3	3	AF532681
00-078-0883	14	14-g	14	
00-074-3370	14	14-g	14	
00-070-0212	23F	23F-g=	23F	
		10A-23F		
00-066-3506	4	4	4	
00-043-0876	19A	19A	19A	
00-036-1378	19F	19F	19F	
00-008-0865	8	8	8	
99-348-3354	6A	6A-ca	6B	
99-338-1052	19F	19F	19F	
99-325-0373	23F	23F-c	23F	AF532678
99-324-1010	4	4	4	
99-404-0191	4	4	4	

99-310-0070	4	4	4	
99-302-1894	9V	9V	9V	
99-293-1704	19A	19A	19A	
99-287-2376	35B	35B		
99-287-2320	35B	35B		
99-287-2298	35B	35B		
99-284-1034	14	14-c	14	
99-276-0568	9V	9V	9V	AF532645
99-242-0442A	6B	6B-q	6B	
99-241-1187A	4	4	4	
99-237-2839	9V	9V	9V	
99-235-2193	4	4	4	
99-226-1026B	7F	7F		
99-221-2755	9V	9V	9V	
99-221-2745A1	23F	23F-g= 10A-23F	23F	
99-221-0278	4	4	4	
99-218-2527	23F	23F-g= 10A-23F	23F	
99-201-1708	3	3	3	
99-196-2909B	10A	10A-23F	23F-NEG	
99-196-2908B	10A	=23F-g 10A-23F=	23F-NEG	
99-196-2882A	10A	23F-g 10A-23F	23F-NEG	
99-196-2880A	10A	=23F-g 10A-23F	23F-NEG	
99-195-0430	14	=23F-g 14-g	14	
99-193-2919A	4	4	4	
99-193-2918B	4	4	4	
99-193-2747B	4	4	4	
99-193-2491A	18C	18C/18B	18C	
99-192-0047B	23F	23F-g= 10A-23F	23F	
99-188-2369A	4	4	4	
99-186-2831	7F	7F		
99-186-1038	14	14-g	14	
99-186-0417	14	14-g	14	
99-184-0894	14	14-g	14	
99-182-1919	4	4	4	
99-180-2653	4	4	4	
99-178-0901	14	14-g	14	
99-177-1060	11A	11A-q		
99-176-1983	18C	18C/18B	18C	
99-173-2956	4	4	4	
99-169-0432	6B	6B-g	6B	
99-159-2018	7F	7F		
99-158-1250	14	14-g	14	
99-157-0650	19F	19F	19F	
99-146-2324	19F	19F	19F	
99-144-1497	22F	22F		
99-134-2273	3	3	3	
99-132-2724	15B	15B-q		
99-132-2558	15B	15B-q		
99-132-2557	15B	15B-q		

99-130-2037	14	14-g	14	
99-110-2820	9N	9N		
99-108-0976	23F	23F-g= 10A-23F	23F	
99-107-0715	14	14-g	14	
99-104-1860	4	4	4	
99-099-0423	19F	19F	19F	
99-095-1044	20	20/13		
99-091-2295	23B	23B	23F-NEG	AF532676
99-090-2551	14	14-g	14	
99-090-2390	3	3	3	
99-090-2387	3	3	3	
99-033-2630	23F	23F-g= 10A-23F	23F	
99-028-0057	7C	7C		
99-011-0311A	4	4	4	
Clinical isolates				
New Zealand				
(ESR) ⁹				
NZSPN00/9	4	4	4	
NZSPN00/42	18C	18C/18B	18C	
NZSPN00/59	5	5-q		
NZSPN00/87	13	13/20		
NZSPN00/88	6B	6B-g	6B	
NZSPN00/91	8	8	8	
NZSPN00/319	18B	18B/18C	18C	-;
				AY163212, AY163228
NZSPN00/366	7F	7F		
NZSPN00/426	3	3	3	
NZSPN00/454	23F	23F-23A= 23A-23F	23F	AF532679
NZSPN00/470	9V	9V	9V	
NZSPN00/480	6A	6A-ca	6B	
NZSPN00/484	23F	23F-g= 10A-23F	23F	
NZSPN00/499	19F	19F	19F	
NZSPN01/162	2	2-q	2	
NZSPN01/243	33F	33F-q	33F/37	-;
				AY163203, AY163219
NZSPN01/393	35F	35F		
NZSPN01/468	11A	11A-q		
NZSPN01/481	16F	16F		
NZSPN01/484	23F	23F-g= 10A-23F	23F	
NZSPN01/490	22F	22F		
NZSPN01/493	9N	9N		
NZSPN01/509	23A	23A-ca	23F-X; 23F-Y-NEG	
NZSPN01/510	12F	12F		
NZSPN01/520	9V	9V	9V	
NZSPN01/531	8	8	8	
NZSPN01/534	3	3	3	
NZSPN01/538	38	38/25F		
NZSPN01/543	10A	10A-q		
NZSPN01/546	4	4	4	
NZSPN01/547	20	20/13		

NZSPN01/548	7F	7F		
NZSPN01/549	1	1	1	
NZSPN01/553	17F	17F-c		
NZSPN01/554	19F	19F	19F	
NZSPN01/555	18C	18C/18B	18C	
NZSPN01/557	19A	19A	19A	
NZSPN01/559	6A	6A-c	6B	
NZSPN01/560	14	14-g	14	
NZSPN01/561	6B	6B-q	6B	
NZSPN00/12	17F	17F-c		
NZSPN00/50	Nonserotypeable	Nonserotypeable-nz		AF532714
NZSPN00/59	5	5-q		
NZSPN00/75	Nonserotypeable	No-amplicon		
NZSPN00/180	9V+14	9V	9V+14	
NZSPN00/221	38	38/25F		
NZSPN00/225	13	13/20		
NZSPN00/242	35F	35F		
NZSPN00/353	18A	18A	18C	AF532659;
NZSPN00/410	33F	33F-q	33F/37	AY163209, AY163225
NZSPN01/93	16F	16F		AF532688;
NZSPN01/122	10A	10A-q		AY163202, AY163218
NZSPN01/146	38	38/25F		
NZSPN01/166	16F	16F		AF532654
NZSPN01/204	35B	35B		
NZSPN01/209	22A	22A		
NZSPN01/240	12F	12F		
NZSPN01/254	35F	35F		
NZSPN01/262	8	8	8	
NZSPN01/276	6A	6A-6B-q	6B	-;
NZSPN01/278	18B	=6B-q		AY163177, AY163187
NZSPN01/291	6B	18B/18C	18C	-;
NZSPN01/303	Nonserotypeable	6B-q	6B	AY163213, AY163229
NZSPN01/313	18C	No-amplicon		
NZSPN01/329	6A	18C/18B	18C	
NZSPN01/335	19A	6A-6B-g	6B	AF532701;
NZSPN01/344	18C	=6B-g		AY163175, AY163185
NZSPN01/361	9N	19A	19A	
NZSPN01/363	18C	18C/18B	18C	
NZSPN01/366	6A	6A-ca	6B	
NZSPN01/369	18C	18C/18B	18C	
NZSPN01/374	35B	35B		
NZSPN01/387	22F	22F		
NZSPN01/388	12F	12F		
NZSPN01/389	20	20/13		
NZSPN01/403	20	20/13		
NZSPN01/411	11A	11A-nz		AF532638
NZSPN01/418	8	8	8	
NZSPN01/428	3	3	3	AF532683
NZSPN01/431	1	1	1	
NZSPN01/437	1	1	1	
NZSPN01/438	22F	22F		

NZSPN01/448	11A	11A-q	
NZSPN01/455	19A	19A	19A
NZSPN01/463	10A	10A-q	
NZSPN01/465	22F	22F	
NZSPN01/477	10A	10A-23F =23F-g	23F-NEG
NZSPN01/478	20	20/13	
NZSPN01/483	8	8	8
NZSPN01/485	12F	12F	
NZSPN01/489	3	3	3
NZSPN01/497	9N	9N	
NZSPN01/505	19A	19A	19A
NZSPN01/512	7F	7F	
NZSPN01/515	3	3	3
NZSPN01/516	1	1	1
NZSPN01/529	1	1	1
NZSPN01/532	4	4	4
NZSPN01/535	7F	7F	
NZSPN01/539	19F	19F	19F
NZSPN01/545	18C	18C/18B	18C
NZSPN01/556	6B	6B-q	6B
NZSPN01/558	14	14-g	14

Notes.

1. CS of selected *S. pneumoniae* isolates from reference panels 1 and 3 was
5 repeated by Gail Stewart and Robert Gange at Department of Microbiology, Children's
Hospital at Westmead, New South Wales, Australia.
2. MCT was performed and GenBank accession numbers generated by Fanrong
Kong at Centre for Infectious Diseases and Microbiology (CIDM), Institute of Clinical
Pathology and Medical Research (ICPMR), Westmead Hospital, Westmead, New
10 South Wales, Australia. See text for molecular capsular subtype (mctsp) nomenclature.
3. Provided by Denise Murphy, Pneumococcal Reference Laboratory, Public
Health Microbiology, Queensland Health Scientific Services, Queensland, Australia.
4. Provided by Associate Professor Geoff Hogg and Jenny Davis, Microbiological
Diagnostic Unit (MDU), Public Health Laboratory, Department of Microbiology and
15 Immunology, University of Melbourne, Victoria, Australia.
5. Provided by Dr. Louise P. Jette, Institut National de Sante Publique du Quebec-
Laboratoire de Sante Publique du Quebec, Sainte-Anne-de-Bellevue, Quebec H9X
3R5, Canada.
6. Provided by Dr. Michael Watson, Department of Microbiology, Children's
20 Hospital at Westmead, New South Wales, Australia.
7. Selected 21 *S. pneumoniae* clinical isolates, of which CS results were known,
from the CIDM diagnostic laboratory.

8. 152 Australian *S. pneumoniae* clinical isolates, of which CS results were known, from the CIDM diagnostic laboratory.
9. 103 New Zealand *S. pneumoniae* clinical isolates Provided by Dr. Diana Martin, from Streptococcus Reference Laboratory, at Institute of Environmental Science and
5 Research (ESR), Wellington, New Zealand.

Conventional serotyping (CS)

CS was performed by the Quellung reaction using rabbit polyclonal antisera
10 from the Statens Serum Institute, Copenhagen, Denmark (Sorensen, 1993). Briefly, 2 μ L of a suspension of isolate, in 10% formalin saline, and 1 μ L of antisera, under a glass coverslip were examined for capsular swelling using a light microscope at 400x magnification. Clinical isolates from CIDM were serotyped at Department of Microbiology, Children's Hospital at Westmead, Sydney, Australia and those from
15 New Zealand by the Streptococcus Reference Laboratory, at ESR, Wellington, New Zealand. Selected New Zealand clinical isolates for which only serogroup results were available and selected isolates from reference panels 1 and 3 were re-tested at Children's Hospital at Westmead.

20 Molecular capsular sequence typing - development of method

Oligonucleotide primers

The oligonucleotide primers used in this study, their target sites and melting temperatures are shown in Table 2 and the primer pair specificities and expected amplicon lengths in Table 3. Primers were designed with high melting temperatures to
25 be used in rapid cycle PCR (Kong et al., 2000).

Four previously published *S. pneumoniae*-specific primers, targeting *psaA* (P1, P2) (Morrison et al., 2000) and pneumolysin (IIa, IIb) (Salo et al., 1995) were modified to give high melting temperatures and used to confirm that isolates were *S. pneumoniae*. Primers were designed to amplify and sequence portion of the *cpsA-cpsB*
30 gene region and to amplify serotype/serogroup-specific sequences in the *wzy* and *wzx* genes of 16 *S. pneumoniae* serotypes for which *cps* gene cluster sequences were available. In order to further explore the sequence heterogeneity, part of the *wzx* and *wzy* genes of isolates belonging to serogroups 6, 18, 23 and 33/37 were also sequenced. For serotype 3, which does not contain *wzy* and *wzx* genes, serotype-specific PCR
35 targeted the *orf2 (wze)-cap3A-cap3B* region (Arrecubieta et al., 1996).

Table 2. Oligonucleotide primers used in this study.

Primer	Target gene	T _m °C ¹	GenBank accession numbers	Sequence ^{2,4}
*P1 ⁵	<i>psaA</i>	72.9	U53509	203TAC ATT ACT CGT TCT CTT TCT TTC TGC AAT CAT TCT TG240 (SEQ ID NO:64)
*P2 ⁵	<i>psaA</i>	72.7	U53509	1066TAG TAG CTG TCG CCT TCT TTA CCT TGT TCT GC1035 (SEQ ID NO:65)
*IIa ⁶	<i>pneumolysin</i>	71.9	M17717	457AGA ATA ATC CCA CTC TTC TTG CGG TTG A484 (SEQ ID NO:66)
*IIb ⁶	<i>pneumolysin</i>	71.4	M17717	680CAT GCT GTG AGC CGT TAT TTT TTC ATA CTG651 (SEQ ID NO:67)
cpsS1 ⁷	<i>cpsA (wzg)</i>	75.4	U09239	1030GGC ATT(C) TAT GGA GTT GAT TCG(A) TCC ATT(C) CAC ACC(T) TTA G1066 (SEQ ID NO:68)
cpsS2 ⁷	<i>cpsA (wzg)</i>	71.9	U09239	1057CAC ACC(T) TTA GAA AAT(C) CTC TAT GGA GTG GAT ATC AAT TAC TAT G1099 (SEQ ID NO:69)
cpsS3 ⁷	<i>cpsA (wzg)</i>	68.7	U09239	1447GAA AGT GGG(A/T) GGG(A/T) A(G)A(C)T(G) TAT(C) AAA GTA(G) AAT TCT(G) CAA GAT(C) TTA(G) AAA(G) G1489 (SEQ ID NO:70)
cpsA1 ⁷	<i>cpsA (wzg)</i>	71.5	U09239	1549CCA TCA C(T)AT AGA GGT TAC(A) TG(A)T CTG GCA TT(C)G C1519 (SEQ ID NO:71)
cpsA2 ⁷	<i>cpsB (wzh)</i>	67.0	U09239	1949T(G)CA TG(A)C TA(G)A AC(T)T CT(A)A TC(T)A AG(A)G CAT AAC GAC TAT C(T)1916 (SEQ ID NO:72)
cpsA3 ⁷	<i>cpsB (wzh)</i>	75.6	U09239	2030GC(T)T CAA TG(A)T GG(A)G CAA TG(T)A CTG GA(C)G TA(G)A TTC CCA(G) ACA TC1993 (SEQ ID NO:73)
1YS	<i>cap1H (wzy)</i>	72.1	Z83335	10289GTA GGT GTA GTT TTT TCA GGG ACT TTA ATT TTA TGC AGT G10328 (SEQ ID NO:74)

1YA	<i>cap1H (wzy)</i>	70.4	Z83335	10584 TCG CTT AAC ACA ATG GCT TTA GAA GGT AGA G10554 (SEQ ID NO:75)
2YS	<i>cps2H (wzy)</i>	70.5	AF026471	9711GTT ATT TTA TTT TTT TTG TCG GCA TTG TAT TCT TTA TAT CG9751 (SEQ ID NO:76)
2YA	<i>cps2H (wzy)</i>	71.3	AF026471	10058CAA ATT CAT CGT TTG TAT CCA TTT AAC TGC ATC10026 (SEQ ID NO:77)
4YS	<i>wzy</i>	70.2	AF316639	9601CTT ATA TCT AAT TAT GTT CCG TCT ATA TTT ATA TGG GTT TGC TTT C9646 (SEQ ID NO:78)
4YA	<i>wzy</i>	71.1	AF316639	9948TTT CTC TTC ATT TTC CTG ATA ATT TTG TAC TTC TGA ATG9910 (SEQ ID NO:79)
6A6BYS0 ⁷	<i>wzy</i>	62.6	AY078347 & AF316640	8196/9186ATG CTT TTA AAT TTC TTA TTC ATA TCT ATT TTT C8229/9219 (SEQ ID NO:80)
6A6BYS	<i>wzy</i>	72.0	AY078347 & AF316640	8264/9254G(/A)GA TTT T(G)TT TCA ACC T(C)GC AGT AAT TTT AAC AA(C/T)C(T)G(A)8298/9288 (SEQ ID NO:81)
6A6BYA	<i>wzy</i>	71.4	AY078347 & AF316640	8578/9568CCT GAA AAC AA(G/T) ACT(/C) ACT TTC TGA ATT TCA C(T)GG A(G/T)A TAA AG8538/9528 (SEQ ID NO:82)
6A6BYA1 ⁷	<i>wzy</i>	72.4	AY078347 & AF316640	8944/9934GTA AAC AGA GAG CGA GTG ATC ATT TTA AAA CTT TTG G8808/9898 (SEQ ID NO:83)
8YS	<i>wzy</i>	70.5	AF316641	10810GTT TTA TTG ACT TTA AAG ATG TTA GTT TCT TCG ATT CCA G10849 (SEQ ID NO:84)
8YA	<i>wzy</i>	70.5	AF316641	11086TTT TTA TTA CTC TTC TTA AAT CAT AAT GAA TCG TAC CAA TCA AC11043 (SEQ ID NO:85)
9VYS	<i>cps9vl (wzy)</i>	73.5	AF402095	8535GGA TCA ATG GCA ACT ATA TTT ACC CTA CTC TCC ACA G8571 (SEQ ID NO:86)
9VYA	<i>cps9vl(wzy)</i>	76.3	AF402095	8872GAG TCG AAA CCA ACC GGA AAA AGC AAT TGA G8842 (SEQ ID NO:87)
14YS	<i>cps14H (wzy)</i>	71.5	X85787	7361CCT TTG GTT TAT TAT CCT ACT TCC AAA ACA GTT TAT GC7398 (SEQ ID NO:88)

14YA	<i>cps14H (wzy)</i>	71.4	X85787	7670CAT ATA TCT CTT TAT CCT GTC AAT ATT GAT TGG CAT TTT C7631 (SEQ ID NO:89)
18CYS0 ⁷	<i>wzx</i>	71.3	AF316642	11856GAA ATT ATA GTC GGA GCT TTC ATT TAT ATT AGT TTA CTG GTT CTG11900 (SEQ ID NO:90)
18CYS	<i>wzy</i>	71.5	AF316642	12190GAT ATT AGC TAT ACC AAC AAT TGT TCT TTT CCT GTA CTC AGT C12232 (SEQ ID NO:91)
18CYA	<i>wzy</i>	72.5	AF316642	12491GCA TTT CTA GTA CCG AAC CAT TGA AAC TAT CAT CTG12456 (SEQ ID NO:92)
18CYA1 ⁷	<i>wzy</i>	73.3	AF316642	12536CAG AAT AAA GAG AGC TGT AAT AGG TGC AAC TTC ATG C12490 (SEQ ID NO:93)
19FYS	<i>cps19fI (wzy)</i>	70.6	U09239	7673CTG TAA TGT TTC TAA TTA GTT CAG TAT TTG CAC TGG TTA ATT C7715 (SEQ ID NO:94)
19FYA	<i>cps19fI (wzy)</i>	72.0	U09239	7958CCC GTA TAT CCA TTA CTA AGA ACA AGG TTG TAT ATT TCC TTC7917 (SEQ ID NO:95)
19AYS	<i>cps19aI (wzy)</i>	71.2	AF094575	9245GTT TCT CAT TAG TTC TGT ATT TGC CCT TAT TAA TGT GC9282 (SEQ ID NO:96)
19AYA	<i>cps19aI (wzy)</i>	72.2	AF094575	9514CCA TGG CTA AGT GCA AGA TTA TGA ATC TCT CTC9482 (SEQ ID NO:97)
19B19CYS	<i>cps19bI (wzy)</i>	71.6	AF004325	3519GTT TCT TAT GTT TAC CCT CAG CTT ATA TTG GCA CAG3554 (SEQ ID NO:98)
19B19CYA	<i>cps19bI (wzy)</i>	71.5	AF004325	3946GAT ACC ACA AAT CTC CGA ATT CTC TTA AAA TAG ATG G3910 (SEQ ID NO:99)
23FYS	<i>cps23fG (wzy)</i>	71.6	AF057294	8567TTA AGT AGT TCA CAA GTG ATA GTG AAC TTG GGA TTG TC8604 (SEQ ID NO:100)
23FYA	<i>cps23fG (wzy)</i>	70.7	AF057294	8846CAC TGA GAT TAT TTA TTA GCT TTA TCG GTA AGG TGG ATA AG8806 (SEQ ID NO:101)
33F37YS0 ⁷	<i>cap33fJ</i>	76.0	AJ006986	11191CCA ATG AAA AGG AAA GTT CAA TGT GTT TTG TTT CTG C11227 (SEQ ID NO:102)

33F37YS	<i>cap33/K & cap37K (wzy)</i>	70.7	AJ006986 & AJ131984	11341/11708ATT ACT TGT AAT ACT ATG TAT TCA ACT AGT CA/(C)A GGA TTT GAT GG11384/11751 (SEQ ID NO:103)
33F37YA	<i>cap33/K & cap37K (wzy)</i>	71.7	AJ006986 & AJ131984	11650/12017GAACAAATTTCCGTATCAGATTTGCGA TTTC11620/11987 (SEQ ID NO:104)
33F37YA1 ⁷	<i>cap33/K (wzy)</i>	72.2	AJ006986	11858GGT GCT TCA GCA AAA ATC CCC GTA TTT CTT ATC AG11824 (SEQ ID NO:105)
1XS	<i>cap11 (wzx)</i>	72.6	Z83335	12017TAG CTG ATG TTC CGA TAA ATT ATG GTG GGG TAA TAA TAG12055 (SEQ ID NO:106)
1XA	<i>cap11 (wzx)</i>	70.6	Z83335	12442CTG CGA CAC TGT ATA TAC CTA CAT TAT AAC TAC TAG ACA TTT GC12399 (SEQ ID NO:107)
2XS	<i>cps2J (wzx)</i>	71.8	AF026471	12167GCA ACT TTG GTT CTA AAA TTT TAG TCT TTT TAA TGG TTC C12206 (SEQ ID NO:108)
2XA	<i>cps2J (wzx)</i>	72.1	AF026471	12595TGT TAA ACC CCA ATA TAG AAA TTG TAT TGA GAA TAG CAG C12556 (SEQ ID NO:109)
4XS	<i>wzx</i>	73.2	AF316639	12119CG TTA ATA GCT TAT GTT CAA CTG GTG ATT GAT TTT GG12155 (SEQ ID NO:110)
4XA	<i>wzx</i>	72.0	AF316639	12442TGA TAG TTT TAG AAA TAA TAT AAG GAA TTG CAA CTG CAT GC12402 (SEQ ID NO:111)
6A6BXS0 ⁷	<i>cpsJ-wzx spacer</i>	72.7	AY078347 & AF246898	9581/4550GGT AGG TAT TTT AAT TGG AGG AAG AGA GTC TTG AAT GG9618/4587 (SEQ ID NO:112)
6A6BXS	<i>wzx</i>	72.5	AY078347 & AF316640	9695/10685TTC ATG TC/(T)T(C) TTT TG/(A)T CTA ATC TGA TTA CAA TTG/(C) TC/(T)A CAT CG/(A)9735/10725 (SEQ ID NO:113)
6A6BXA	<i>wzx</i>	74.1	AY078347 & AF316640	9999/10989T/(C)GC ATT TG/(T)G ATC TGT CAC AA/(G)T CAA TAA GTT AAA ACC9964/10954 (SEQ ID NO:114)
6A6BXA1 ⁷	<i>wzx</i>	72.5	AY078347 & AF246898	10682/5651ATC TTC CCT TCA TAA ATT GAC ATA GGA AAA ATA AGA GCC10644/ 5613 (SEQ ID NO:115)

8XS	wzx	71.8	AF316641	8602CAA TTC TAA CTA TGT CCA GTT TTA TTT TTC CAC TCA TCA G8641 (SEQ ID NO:116)
8XA	wzx	74.2	AF316641	8926GAC GTG ATA ATA AGC TGC CAT TCC TGT CTA AAA CG889 (SEQ ID NO:117)
9VXS	<i>cps9vK</i> (wzx)	74.5	AF402095	10543CGG CGG TAT TAA GTA GAA TAT TAA CAC CTG AAG AGT ATG GC10583 (SEQ ID NO:118)
9VXA	<i>cps9vK</i> (wzx)	73.6	AF402095	10910GGC AAT CAG ACT CAA TAA GTT CAT CCG TTT AAA GTT C10874 (SEQ ID NO:119)
14XS	<i>cps14L</i> (wzx)	72.1	X85787	11463GGT ATT GCC TTT CCT TTG ATA ACT TCT CCT TAT TTA TCA C11502 (SEQ ID NO:120)
14XA	<i>cps14L</i> (wzx)	71.6	X85787	11751TGA ACT TGT AAC TCG ACA CCC AAA AAT ATA AAT AAA TGA G11712 (SEQ ID NO:121)
18XS0 ⁷	<i>wciW</i>	75.0	AF316642	10403CAA AGG AAC GTT ATC AGC AAT TGT GTC AAA TTT CAG10438 (SEQ ID NO:122)
18XS	wzx	72.5	AF316642	10715GAA TCG GAC AAT AGC ACA GGT ACG AAC AAG10744 (SEQ ID NO:123)
18CXA	wzx	75.2	AF316642	11082GCC ATG TAA TCA ACT GAC CAA GCA GGG TAC TC11051 (SEQ ID NO:124)
18CXA1 ⁷	wzx	72.2	AF316642	11123AAG ATT AGG GCG CAC AAA GTT TAC TTG TTT TAG C11090 (SEQ ID NO:125)
19FXS	<i>cps19fJ</i> (wzx)	71.3	U09239	8975GTT ATT TCT TCA AAT CTG CTC ATA GTT TTA ACC TCA TCA C9014 (SEQ ID NO:126)
19FXA	<i>cps19fJ</i> (wzx)	73.5	U09239	9279TAT CTT GCG TTT TCA TCC CTT ACA GTT ATT AGG TTC AAA G9240 (SEQ ID NO:127)
19AXS	<i>cps19aJ</i> (wzx)	74.7	AF094575	10547TTC TTC AAA TCT TTT GAC AGT CTT GAC CTC TTC CTT G10583 (SEQ ID NO:128)
19AXA	<i>cps19aJ</i> (wzx)	72.3	AF094575	10846TAT CGT GCA TTC GAA TCT GTT ACA GCT AAT ACA TTT AAA C10807 (SEQ ID NO:129)
19B19CXS	<i>cps19bJ</i> (wzx)	74.3	AF004325 & AF105116	7778/373GTC CTG ACG CTA TCA AAT ATC ATT TTC CCA TTA ATC AC7815/410 (SEQ ID NO:130)

19B19CXA	<i>cps19bJ (wzx)</i>	73.2	AF004325 & AF105116	8104/699CCC ACA TGT GAT CAA TAG GAG TGA AAA TTC TCT ATT C8068/663 (SEQ ID NO:131)
23FXS0 ⁷	<i>cps23fI</i>	73.4	AF057294	11714CCT TTG GCT AAT TTC TTG GAC GAT AAT GAA TTT GTA TAT G11753 (SEQ ID NO:132)
23FXS	<i>cps23fJ (wzx)</i>	72.3	AF057294	11961GCT TTG GCT AAC TTT TCA TCA AAG ATT TTA ATT TTT TTG TTA G12003 (SEQ ID NO:133)
23FXA	<i>cps23fJ (wzx)</i>	73.3	AF057294	12361CCA GAG ATA GCT GTA ACA CCA ATT TTA TCA ATT CCC TTA G12322 (SEQ ID NO:134)
23FXA1 ⁷	<i>cps23fJ (wzx)</i>	72.5	AF057294	12457CCA CAA ACA TTA GCA ATA AAG AAA CCT AAC AAT CCC12422 (SEQ ID NO:135)
33F37XS0 ⁷	<i>cap33fK (wzy)</i>	76.7	AJ006986	12271GTT GTT TTA GCT CAA GGA GGG ATA ATG TTG GCT TCG12306 (SEQ ID NO:136)
33F37XS	<i>cap33fI & cap37L (wzx)</i>	72.2	AJ006986 & AJ131984	12591/12958GAT CAT ACT CCC TAT CAT TAC GAC TCC CTA TGT AAC G12627/12994 (SEQ ID NO:137)
33F37XA	<i>cap33fI & cap37L (wzx)</i>	72.1	AJ006986 & AJ131984	12918/13285CCA AGA AAT ATC CAA ACC TTT TGA CAC TAA ACT TAA TCC12880/13247 (SEQ ID NO:138)
33F37XA1 ⁷	<i>cap33fL (wzx)</i>	73.3	AJ006986	13016GCT GAT TTT ACA AAT AGG AAA ATA GAG ATT GCA CCA AC12979 (SEQ ID NO:139)
3S1	<i>orf2 (wze)- cap3A spacer</i>	72.6	Z47210	5793GCA CAA AAA AAA GTT TGA TAT TCC CCT TGA CAA TAG5828 (SEQ ID NO:140)
3A1	<i>cap3A</i>	73.3	Z47210	6113GCA GGA TCT AAG GAG GCT TCA AGA TTC AAC TC6082 (SEQ ID NO:141)
3S2	<i>cap3A</i>	72.4	Z47210	6933CGA ACC TAC TAT TGA GTG TGA TAC TTT TAT GGG ATA CAG AG6973 (SEQ ID NO:142)
3A2	<i>cap3B</i>	75.7	Z47210	7229CTG ACA GCA TGA AAA TAT ATA ACC GCC CAA CGA ATA AG7192 (SEQ ID NO:143)

Notes.

1. Primer T_m values provided by the primer synthesiser (Sigma-Aldrich).

2. Numbers represent the numbered base positions at which primer sequences start and finish (starting at point “1” of the corresponding gene GenBank sequence).
3. Underlined sequences show bases added to modify previously published primers.
4. Letters in parentheses indicate alternative nucleotides in different serotypes.
- 5 5. Morrison, et al. 2000.
6. Salo, et al. 1995.
7. For sequencing use only.
- * Primers have been previously published. All others primers designed specifically for this study.

Table 3. Specificity and expected lengths of amplicons of primer pairs used in this study.

Primer pairs¹	Specificity	Length of amplicons (base pairs)
P1/P2	<i>S. pneumoniae</i>	864
IIa/IIb	<i>S. pneumoniae</i>	224
cpsS1/cpsA3 ²	<i>S. pneumoniae</i>	1001
cpsS1/cpsA1 ²	<i>S. pneumoniae</i>	520
cpsS3/cpsA2 ²	<i>S. pneumoniae</i>	503
1YS/1YA	serotype 1	296
2YS/2YA	serotype 2	348
4YS/4YA	serotype 4	348
6A6BYS/6A6BYA	serogroup 6	315
6A6BYS0/6A6BYA1 ²	serogroup 6	747
8YS/8YA	serotype 8	277
9V9AYS/9V9AYA	serotypes 9V and 9A	338
14YS/14YA	serotype 14	310
18CYS/18CYA	serogroup 18	302
18CYS0/18CYA1 ²	serogroup 18	671
19FYS/19FYA	serotype 19F	286
19AYS/19AYA	serotype 19A	270
19B19CYS/19B19CYA	serotypes 19B and 19C	428
23FYS/23FYA	serotype 23F	280
33F37YS/33F37YA	serotypes 33F/33A/37	310
33F37YS0/33F37YA1 ²	serotypes 33F/33A/37	668
1XS/1XA	serotype 1	426
2XS/2XA	serotype 2	429
4XS/4XA	serotype 4	324
6A6BXS/6A6BXA	serogroup 6	305
6A6BXS0/6A6BXA1 ²	serogroup 6	1102
8XS/8XA	serotype 8	325
9V9AXS/9V9AXA	serotypes 9V and 9A	368
14XS/14XA	serotype 14	289
18CXS/18CXA	serogroup 18	368
18CXS0/18CXA1 ²	serogroup 18	721
19FXS/19FXA	serotype 19F	305

19AXS/19AXA	serotype 19A	300
19B19CXS/19B19CXA	serotypes 19B and 19C	327
23FXS/23FXA	serotypes 23F/23A	401
23FXS0/23FXA1 ²	serotypes 23F/23A	744
33F37XS/33F37XA	serogroups 33/37	328
33F37XS0/33F37XA1 ²	serotypes 33F/33A/37	746
3S1/3A1	serotype 3	321
3S2/3A2	serotype 3	297

Notes.

1. See Table 2 for primer sequences.
2. For sequencing use only.

5

DNA preparation, PCR and sequencing

DNA extraction, PCR and sequencing were performed as previously described (Kong et al., 2002).

10

Sequence comparison, multiple sequence alignments, and phylogenetic analysis

Sequences were compared using Bestfit in Comparison program group. Multiple sequence alignments were performed with Pileup and Pretty in Multiple Sequence Analysis program group. Phylogenetic relationships were studied using Ednadist and Ekitsch in Evolutionary Analysis program group. All programs are provided in WebANGIS, ANGIS (Australian National Genomic Information Service), 3rd version.

15

Nucleotide sequence accession numbers

The new partial sequence data for *cpsA-cpsB*, *wzy* (polymerase) and *wzx* (flippase) genes for selected reference and clinical isolates reported in this paper have appeared in the GenBank Nucleotide Sequence Databases, with accession numbers AF532632-AF532715, and AF163171-AF163232, respectively (Table 1).

20

Previously reported sequence data used in this paper, in addition to those listed in Table 2, have appeared in GenBank Nucleotide Sequence Databases with the following accession numbers: U15171, U66846 and U66845 (*cps* gene cluster for serotype 3); NC_003028 (serotype 4 genome); AJ239004 (*cps* gene cluster for serotype 8); AF030367-AF030372 (*cps* gene cluster for serotype 19F); AF105113 (partial *cps* gene cluster for serotype 19A); AF105114 and AF106137 (partial *cps* gene

25

clusters for serotype 19B); AF105115 (partial *cps* gene clusters for serotype 19C); AF030373 and AF030374 (*cps* gene clusters for serotype 23F).

RESULTS

5 Both pairs of *S. pneumoniae* species-specific primers (targeting *psaA* and pneumolysin genes) produced amplicons of the expected size from all reference and clinical isolates except six of 179 CIDM isolates, which, on retesting, were optochin resistant and therefore excluded from further study as they were not *S. pneumoniae*.

10 The sequencing primers, *cpsS1/cpsA3*, formed amplicons from all but 13 reference and clinical isolates. Of these 13 isolates, 10 (eight belonging to serotypes 38/25F and two that were nonserotypable) formed amplicons with primer pairs *cpsS1/cpsA1* and *cpsS3/cpsA2*. Three nonserotypable isolates did not form amplicons using any of the primer pairs targeting the *cpsA-cpsB* region, although they had been confirmed to be *S. pneumoniae* using both species-specific PCR.

15 Sequence heterogeneity in the region between the 3'-end of *cpsA* and the 5'-end of *cpsB*

The present inventors sequenced and analyzed 800 bp fragments of the region between the 3'-end of *cpsA* (starting at base pair 951) and the 5'-end of *cpsB* (see 20 Figure 2). Representative sequences were deposited into GenBank (see Table 1 for accession numbers). There were 424 sites that were identical for all 51 serotypes represented among the isolates examined, leaving 376 (47%) heterogeneity sites.

Intra- and inter-serotype/subtype heterogeneity

25 Only single isolates were available for 11 serotypes and the mixed serotype 9V/14 (see below). Among 40 serotypes, for which multiple isolates were available, 14 were divided into molecular capsular sequence types, on the basis of major and/or stable intra-serotype heterogeneity. Molecular capsular sequence types were named according to their conventional serotype (cs) and, generally, the source of the isolate in 30 which the sequence difference was first identified [-g = Genbank sequence; -c (CIDM); -q (Queensland); -ca (Canada); -nz (New Zealand)]. When sequences characteristic of two serotypes were present in the *cpsA-cpsB* region subtype names included both, with the CS first (e.g 23F-23A when CS was 23F; 23A-23F when CS was 23A). Seventeen serotypes had no intra-serotype heterogeneity and in nine there were minor and/or less 35 stable variations between isolates and/or between sequences disclosed herein with corresponding sequences in GenBank (Table 4, Figure 2).

Table 4. Molecular capsular type (MCT) heterogeneity sites in the region between the 3'-end of *cpsA* and the 5'-end of *cpsB* of 51 *S. pneumoniae* serotypes.

MCT (n=) ^a	Intra-MCT ^b Heterogeneity Site - base	Identity between MCT (%)	MCT ^b -specific heterogeneity site - base	Selected heterogeneity sites shared with other MCT ^b - base
1 (9+g)	133 - T ^g /A ^g		289 - A, 452 - A	122 - T, 152 - A, 495 - A, 600 - A
2-g (g)	-		705, 706 - CG	287 - G, 507 - G, 534 - A
2-q (3)	Nil	95.9%	239 - C, 293 - T, 386 - A, 404 - G	232 - G, 286 - C, 600 - A
3 (17+g)	262 - C ^{g+16} /T ¹ , 292 - G ¹⁶ /A ^{g+1} , 293 - A ¹⁶ /G ^{g+1} , 539 - C ¹⁶ /T ^{g+1} , 545 - C ^{g+16} /A ¹		485 - A, 487 - A	27 - A, 90 - A, 231 - A, 590 - T, 686 - T
4 (36)	Nil		179 - C	231, 232 - TG, 611 - T, 743 - T
5-q (4)	Nil			428 - T, 599 - A
5-c (1)	-	94.0%		122 - T, 152 - A, 247 - C, 605 - T
6A-g (g)	463-5 - AGC ¹² /GCA ^g , 534 - A ^g /G ¹² , 542 - C ^g /T ¹² , 545 - A ^g /C ¹²			62 - A, 209 - A, 534 - A, 542 - C
6A-ca (7)	55 - A ⁵ /G ² , 331 - A ² /G ⁵ , 434 - A ⁵ /G ²	6A-ca : 6A-g=99.1%		62 - A, 209 - A
6A-c (2)	Nil	6A-c : 6A-ca=99.5%		62 - A, 209 - A, 337 - G
6A-6B-g (2)	(see 6B-g) 772 - A ^{g+1} /G ¹			(see 6B-g)
6A-6B-q (1)	(see 6B-q)			(see 6B-q)

6B-g (4+g)	31 - A ¹ /G ^{g+3}			209 - A, 337 - G, 341 - G,
6B-q (9)	383 - A ⁸ /G ¹	6B-q : 6B-g=84.7%	749 - G	52 - G, 58 - C, 68 - G, 82 - C, 85 - T, 94 - T, 104 - T, 116 - G, 160 - T, 209 - C, 286 - C, 343 - G, 375 - G, 478 - C, 490 - C, 521 - T, 563 - T, 704 - C, 776 - C
6B-c (1)	-	6B-c : 6B-g=92.1%		193 - T, 209 - C
7F (15)	Nil		66 - C, 445 - C	722 - C, 731 - A
7C (3)	Nil			49 - C, 731 - A
8 (12)	Nil		340 - T, 670 - G	425 - A
9N (9)	Nil		81 - T, 378 - A	352 - G, 409 - T, 590 - T, 722 - A
9V (17)	Nil		245 - G	428 - C, 704 - C, 750 - T, 776 - C
10F (2)	309 - G ¹ /A ¹ , 335 - G ¹ /A ¹			704 - C, 750 - T, 776 - C
10A-q (5)	Nil		222 - T, 663 - T	232 - G
10A-23F (6)	(see 23F-g)	91.2%		(see 23F-g)
11A-q (7)	Nil			122 - T, 232 - G, 478 - C, 490 - C, 521 - T, 704 - C
11A-nz (1)	-	94.0%	316 - T	597 - A
11B (1)	-		269 - A, 490 - G, 776 - T	10 - G, 52 - G, 58 - C, 68 - G, 82 - C, 85 - T, 94 - T, 104 - T, 116 - G, 148 - T, 160 - T, 231, 232 - TG, 247 - C, 250 - A, 286 - C, 292 - C, 343 - G, 375 - G, 425 - A, 521 - T, 563 - T, 704 - C
12F (9)	268 - A ¹ /C ⁸ , 572 - C ¹ /T ⁸ , 781 - G ¹ /T ⁸		274 - C	287 - G, 497 - G, 577 - T, 722 - C

13 (6)/20 (8)	Nil; Nil			590 - T, 686 - T, 722 - A
14-g (32+g)	249 - T^{23}/C^{g+9} , 250 - G^{32}/T^g , 320 - G^{32}/A^g			577 - T
14-c (1)	-	98.1%	613 - G	16 - C, 49 - C, 54 - T, 62 - T, 406 - G, 577 - T
15A-ca1 (1)	-		473 - G	49 - C, 337 - G, 507 - G
15A-ca2 (1)	-	95.1%	406 - A, 473 - G	337 - G, 507 - G
15B-q (5)	Nil			232 - G
15B-c (1)	-	15B-c : 15B-q=97.4%	235 - T, 351 - G	49 - C, 247 - C, 352 - G, 428 - T, 542 - C
15B-22F (2)	(see 22F)	15B-22F : 15B-q=95.2%		(see 22F)
15C-q (1)	as for 15B-q plus 104 - T^C/C^B			232 - G
15C-CA (1)	as for 15B-q plus 232 - A^C/G^B , 757 - T^C/C^B	99.6%		pattern
16F (6)	149 - C^5/T^1 , 232 - A^5/G^1			122 - T, 232 - G, 352 - G, 548 - A
17F-c (3)	Nil			199 - A, 247 - C, 600 - C
17F-35B (2)	(see 35B)	99.8%	728 - C	(see 35B)
17A (1)	-		122 - A	85 - T, 554 - G, 567 - A
18F (1)	-		65 - A, 161 - T, 469 - C, 684 - A	722 - C, 786 - C
18A (2)	63 - T^1/A^1		99 - C, 202 - G, 232 - C, 239 - G, 322 - C, 334 - C,	122 - T, 307 - G, 563 - T, 686 - T

18B (4)/18C (14)	Nil; Nil	138 - G, 459 - C, 478 - C 750 - A	
19F (20+gx7)	164 - C gx^{7+17}/T^3 , 169 - C gx^{6+11}/T^{g+9} , 387 - A gx^{6+20}/T^g , 414 - G gx^{5+20}/T^{gx2} , 479 - G gx^{7+16}/A^4	169 - T, 337 - G	
19A (11+g)	70 - T ^g /C ¹¹ , 479 - A ⁸ /G ^{g+3}	202 - C	49 - C, 54 - T, 62 - T, 94 - A, 103 - C, 104 - T, 160 - T, 198 - C, 232 - G, 286 - C, 343 - G, 352 - G, 375 - T, 425 - A, 490 - C, 750 - T 428 - C, 548 - A, 629 - T, 717 - A 3 428 - T, 567 - G, 599 - A, 731 - A 428 - T, 567 - A, 599 - A, 731 - A 193 - T 249 - A, 337 - G 495 - A 247 - C, 495 - A (as for 23F-23A) 49 - C, 55 - T, 58 - C, 62 - T, 103 - C, 104 - T, 160 - T, 198 - C, 223 - G, 232 - G, 249 - T, 286 - C, 292 - C, 343 - G, 375 - G, 376 - G, 425 - A, 490 - C, 521 - T, 563 - T, 704 - C
21 (1)	-		
22F (13)	Nil		
22A (4)	Nil		
23F-g (17+gx3)	Nil		
23F-c (1)	-	23F-c : 23F-g=91.2% 88 - G	
23F-23A (1)	-	23F-23A : 23F-g=98.7%	
23A-ca (2)	Nil		
23A-23F (1)	(as for 23F-23A)	96.6%	
23B (1)	-	734 - C, 763 - G	

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25F (1)/38 (7)	-; Nil		Numerous sites	Numerous sites
29 (1)	-		310 - A	335 - A
31 (2)/42 (1)	Nil; -			122 - T, 152 - A, 605 - T
33F-g (2+g)/33A (1)	534 - A ^g /G ² ; -			247 - C, 600 - A, 728 - T
33F-q (4)	313 - T ¹ /G ³	94.7%	313 - T	169 - T, 717 - A
33B (1)	-		578 - G	169 - T, 717 - A
34 (2)	Nil			85 - C, 122 - C, 554 - G, 567 - A
35F (6)	Nil			232 - G, 343 - G, 554 - G, 577 - T
35B (9)	Nil			199 - G, 247 - C, 600 - A, 728 - C
37 (1+g)	231 - A ^g /C ¹		54 - G	90 - A, 231 - A, 743 - T
41F (1)	-			287 - G, 507 - G

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Notes.

- Key to mst: -g = Genbank sequence; -c (CIDM); -q (Queensland); -ca (Canada); -nz (New Zealand)
- The superscript numbers = number of isolates studied; superscript g = base present in corresponding GenBank sequence

There were 368 heterogeneity sites that allowed differentiation between molecular capsular sequence types, including both specific and shared sites (Table 4, Figure 2).

5 Phylogenetic tree based on region of the 3'-end of *cpsA*-the 5'-end of *cpsB* genes

Using these 800bp sequences, a phylogenetic tree was inferred for the 132 (included the new sequences from Example 2) *S. pneumoniae* molecular capsular sequence type analysis of the *cpsA-cpsB* region (Figure 3 - it should be noted that in Figure 3 the sequence types were renamed based on serotype and their GenBank
10 accession numbers). Typical class I serotypes (e.g. 1, 18C, 19F), a typical class II serotype (e.g 33F, represented by 33F-g) and a nontypical class II serotype (19A) were each in different clusters of the tree (Jiang et al., 2001).

The phylogenetic tree provides evidence for, and suggests possible sources of, recombination between *cpsA-cpsB* genes of classes I and II. For example, subtype 23F-
15 c (or 23F-AF532678) clustered with 15A-c2 (or 15A-AF532647), but in a separate cluster from other 23F and 15A subtypes, suggesting that they may have arisen by recombination between 23F and 15A, respectively, and other serotypes.

Molecular capsular sequence typing based on *cpsA-cpsB* region sequences

20 The molecular capsular sequence type, assigned on the basis of *cpsA-cpsB* sequence, was the same as the CS for all isolates belonging to 36 of 51 serotypes (or 304 of 394 [77%] isolates), and for the majority of isolates (25 of 39) belonging to another five serotypes (Table 5). The remaining isolates in these serotypes shared sequences with other serotypes, namely 6A with 6B, 10A and 23A with 23F, 15B with
25 22F and 17F with 35B, presumably as a result of recombination. There were five serotype pairs, represented by 46 isolates, whose members had identical sequences: namely 20/13, 18C/18B, 38/25F, 31/42 and 33F-g/33A.

Table 5. Comparison of molecular capsular typing (MCT) and conventional serotyping (CS) results of 394 *S.pneumoniae* isolates.

CS	N=	MCT-seq: a) <i>cpsA-cpsB</i> or b) <i>wzx</i> , <i>wzy</i> type(s) (n) ¹	MCT-PCR (<i>wzy</i> & <i>wzx</i>)	Final MCT	Comment
1	9	1	1	1	Correlate
2	3	2	2	2	"
3	17	3	3	3	"
4	36	4	4	4	"
5	5	5	NA	5	"
6A	12	6A(9); 6B-g (2); 6B-q (1) 6A (11) ² ; 6B-q (1)	Serogroup 6	6A (11) 6B (1)	1 of 12 results discrepant ² 56
6B	15	6B	Serogroup 6	6B	Correlate
7C	3	7C	NA	7C	"
7F	15	7F	NA	7F	"
8	12	8	NA	8	"
9N	9	9N	NA	9N	"
9V	17	9V	9V	9V	"
9V/14	1	9V	9V/14	9V/14	See text
10A	11	10A (5); 23F-g (6) ³	23F <i>wzy/wzx</i> PCR negative (6) ³	10A (11) ³	Correlate ³
10F	2	10F	NA	10F	Correlate
11A	8	11A	NA	11A	"
11B	1	11B	NA	11B	"
12F	9	12F	NA	12F	"

13	6	13/20	NA	13/20	Consistent
14	33	14	14	14	Correlate
15A	2	15A	NA	15A	Correlate
15B	8	15B (6); 22F (2)	NA	15B (6); 22F (2)	2 of 8 results discrepant
15C	2	15C	NA	15C	Correlate
16F	6	16F	NA	16F	"
17A	1	17A	NA	17A	"
17F	5	17F (3); 35B (2)	NA	17F (3); 35 (2)	2 of 5 results discrepant
18A	2	18A	Serogroup 18	18A	Correlate
18B	4	18C/18B	"	18B/C	Consistent
18C	14	C/18B	"	18B/C	"
18F	1	18F	"	18F	Correlate
19A	11	19A	19A	19A	"
19F	20	19F	19F	19F	"
20	8	13/20	NA	20	Consistent
21	1	21	NA	21	Correlate
22A	4	22A	NA	22A	"
22F	13	22F	NA	22F	"
23A	3	23A (2); 23F-g (1) 23A (3) ⁴	23F wzy PCR negative/23F wzz PCR positive ⁴	23A ⁴	" ⁴
23B	1	23B	NA	23B	"

23F	20	23F	23F	23F	“	
25F	1	25F/38	NA	25F/38	Consistent	
29	1	29	NA	29	Correlate	
31	2	31/42	NA	31/42	Consistent	
33A	1	33A/33F-g ⁵	Serogroup 33/37 ⁵	33A/33F ⁵	“ ⁵	
33B	1	33B	Serogroup 33/37 PCR (wzy) negative ⁶	33B	Correlate ⁶	
33F	6	33A/33F-g ⁵ , 33F-q	Serogroup 33/37 ⁵	33A/33F ⁵	Correlate ⁵	
34	2	34	NA	34	Correlate	
35B	9	35B	NA	35B	“	
35F	6	35F	NA	35F	“	
37	1	37	Serogroup 33/37	37	“	58
38	7	25F/38	NA	25F/38	Consistent	
41F	1	41F	NA	41F	Correlate	
42	1	31/42	NA	31/42	Consistent	
Nonserotypable	5	Non-typable ⁷	NA ⁷	Non-typable ⁷	Correlate ⁷	
TOTAL	394				Results:	
					Correlate = 343	
					Consistent = 46	
					Discrepant = 5	

Notes.

1. For nomenclature, see Table 4 and text.
2. *cpsA-cpsB* sequence 3 discrepancies; 2 resolved by *wzx*, *wzy* gene sequences.
3. Six serotype 10A isolates shared *cpsA-cpsB* sequence with 23F-g, but 23F specific PCR (targeting both *wzy* and *wzx*) was negative;
- 5 10A-23F was identified by exclusion of 23F in our existing database. However, this relationship needs to be confirmed by examination of
larger collection isolates.
4. *cpsA-cpsB* sequence 1 discrepancy; resolved by *wzx* gene sequence; 23F *wzx* PCR positive/23F negative *wzy* PCR negative also
support its identification by exclusion.
5. For one serotype 33A isolate, *cpsA-cpsB* and *wzx* and *wzy* sequences were identical with 33F-g but different from 33F-q; 33F/37
10 *wzx* and *wzy* PCR were both positive.
6. One serotype 33B strain identified by exclusion: 33F/37 *wzx* PCR positive/33/37 *wzy* PCR negative.
7. All isolates confirmed to be *S. pneumoniae*. These isolates may belong to rare serotypes not represented among our reference
isolates.

Molecular capsular sequence typing based on PCR targeting *wzy* and *wzx* (*orf2* [*wze*]-*cap3A-cap3B* for serotype 3)

There is significant sequence heterogeneity in *wzy* and *wzx* (data not shown), which made them suitable PCR targets for serogroup or serotype identification (Tables 2 and 3). With few exceptions, primer pairs targeting these genes formed amplicons only from the corresponding serotypes represented in the five reference panels. Exceptions were: PCR targeting serotype 6B also amplified 6A; PCR targeting 18C amplified all serotypes in serogroup 18; PCR targeting *wzx* (but not *wzy*) of serotype 23F, amplified three serotype 23A strains; PCR targeting *wzx* and *wzy* of serotypes 33/37 amplified a 33A isolate and that targeting *wzx* amplified a serotype 33B isolate.

The specificity of serotype 3-specific primers targeting the *orf2* (*wze*)-*cap3A-cap3B* genes (Arrecubieta et al., 1996) was confirmed by production of an amplicon of the expected size from all 17 serotype 3 isolates. Thus, a serotype or serogroup was assigned by PCR to all 239 isolates belonging to serotypes/serogroups for which specific PCR was developed (Table 5).

Comparison of molecular capsular sequence typing based on *cpsA-cpsB* sequencing and PCR/sequencing targeting *wzx* and *wzy*

The results of PCR and *cpsA-cpsB* sequencing were consistent except that PCR could not distinguish between some members of serogroups 6, 18, 23 and 33/37 and further sequencing (of *wzx*, *wzy*) was required to identify individual molecular capsular sequence types (see below). The *cpsA-cpsB* sequences of six 10A isolates were identical to those of 23F, but the isolates were negative in the 23F-specific PCR targeting *wzx* and *wzy* (10A-23F).

Relationships within serogroups

Sequence analysis of the *cpsA-cpsB* region and *wzy* and *wzx* genes (data not shown) showed variable phylogenetic relationships between members of different serogroups.

Serogroup 6

Serotypes 6A and 6B were divided into five and three subtypes, respectively, based on different sequence patterns in the *cpsA-cpsB* region. Three 6A isolates had sequences in this region characteristic of serotype 6B (Table 4). Serotypes 6A and 6B could not be distinguished by PCR targeting *wzx* and *wzy*. Sequencing of these genes correctly identified all except one 6A isolates, but some 6A and 6B subtypes share

identical or very similar sequences. The serotype of the discrepant isolate (serotype 6A, 6B-q) was checked independently by two laboratories (Vakevainen et al., 2001).

Serogroup 18

5 Serotypes 18C and 18B had identical *cspA-cpsB* region sequences and were close to 18A and 18F in the class I cluster (Figure 3). PCR targeting both *wzx* and *wzy* genes amplified all four serotypes. Sequences of 18C and 18B were identical to each other, but different from those of serotypes 18A and 18F, which were also distinguishable from each other.

10

Serogroup 23

Serotypes 23F, 23A (except 23F-23A and 23A-23F) and 23B were separated into different clusters based on *cpsA-cpsB* sequence differences. Serotype 23A (including 23A-23F) was identified on the basis of a positive result with 23F-specific primers targeting *wzx* and a negative result with the corresponding *wzy* PCR. Sequencing could differentiate individual serotypes (23A, 23F and 23B) except 23F-23A and 23A-23F. Mct 23F-c, 23A-23F and 23F-23A have apparently arisen by recombination between 23F, 23A and/or others, producing sequences in the *cpsA-cpsB* regions that are quite different from their parental types.

20

Serogroups 33 and 37

Serotypes 33A and 33F-g share identical *cpsA-cpsB* sequences and that of 33B is similar; 37 and 33F-g cluster together, as do 33B and 33F-q (Figure 3). The 33F/37-specific *wzx* PCR amplified 37, 33F, 33A and 33B, indicating similarities at that site, although sequencing showed clear differences between 33B and the others. The 33F/37-specific *wzy* PCR amplified 37, 33F and 33A but not 33B. Thus, mct 33B was identified on the basis of a positive result with 33F/37-specific primers targeting *wzx* and a negative result with the corresponding *wzy* PCR.

30 *Other serogroups*

Despite antigenic similarities that determine their membership of the same serogroup, serotypes 9N and 9V appear to be genetically distant, on the basis of significant differences between their *cpsA-cpsB* sequences and the fact that 9V-specific PCR did not amplify 9N.

35 Similarly, mct 19F and 19A had quite different *cpsA-cpsB* region sequences and separated into different clusters. 19F-specific PCR did not amplify 19A and vice versa.

There were differences between mct 19F, 19A, 19B, 19C in *wzx* and *wzy* sequences (except *wzy* sequence of 19C was not available in GenBank), but they formed two groups - 19F, 19A and 19B, 19C.

Serotypes 7F and 7C separated into different clusters based on *cpsA-cpsB* sequences, as did 11A and 11B (Figure 3). Serotypes 15B and 15C had similar *cpsA-cpsB* sequences and clustered together, except for 15B-22F. Serotypes 17F (including 17F-c and 17F-35B) and 17A were clustered together. Serotypes 35F and 35B are closely related based on similar *cpsA-cpsB* sequences.

10 Mixed culture

One clinical isolate identified as serotype 9/14 using antisera was positive in 9V- and 14-specific PCR (targeting both *wzx* and *wzy*), but was identified as mct 9V by sequencing. The isolate was subcultured and 16 individual colonies were rested. All 16 colonies were positive in both mct 9V-specific and negative in both 14-specific PCR assays and were identified as mct 9V by sequencing. The serotype of the original isolate was rechecked and the results (mixed serotype 9/14) were as before. It was therefore assumed that the original isolate was a mixture, predominantly of serotype 9V with a minor component of serotype 14.

20 Comparison of serotype identification results between molecular capsular sequence typing and CS

After CS and molecular capsular sequence typing had been completed, the results were compared. Initial results were discrepant for 29 isolates; repeat serotyping and/or correction of clerical errors resolved all but five discrepancies. Final results correlated between CS and molecular capsular sequence typing methods for all isolates of 38 serotypes (318 isolates), 20 of 25 of another three serotypes and all five nonserotypable isolates (total 343 isolates). In addition, there were 46 isolates belonging to pairs of serotypes whose members could not be distinguished from each other by molecular capsular sequence typing but all were assigned to the pair that included the serotype to which they had been assigned by CS. These results were classified as consistent.

The five discrepant results were: one isolate of serotype 6A was identified as 6B-q, two isolates of serotype 15B were identified as 22F and two isolates of serotype 17F as 35B.

Algorithm for serotype assignment of *S. pneumoniae* by molecular capsular sequence typing

An algorithm for practical use of the molecular capsular sequence typing method for the identification of *S. pneumoniae* serotypes is shown in Table 6.

5

DISCUSSION

Sequences of 16 *cps* gene clusters showed that all have the same four genes at their 5' ends - *cpsA* (*wzg*)-*cpsB* (*wzh*)-*cpsC* (*wzd*)-*cpsD* (*wze*) - which are the sites for recombination events that generate new forms of capsular polysaccharide. The sequences for different serotypes can be divided into two classes and show evidence of interesting recombination patterns.

The study of 51 serotypes, of which 40 were represented by more than one isolate, showed that the *cpsA-cpsB* sequences for the same serotypes were generally stable or could be consistently divided into a small number of subtypes. This shows that sequence patterns in this region can be used to identify different serotypes/serosubtypes.

It has been shown previously that PCR-RFLP based on the *cpsA-cpsB* region can predict *S. pneumoniae* serotypes (Lawrence et al., 2000). However, the method generates a long amplicon (1.8kbp), requires the use of three restriction enzymes and special equipment and has limited discriminatory ability.

The present inventors identified 376 sequence heterogeneity sites, in the *cpsA-cpsB* region, among the 51 serotypes studied (Table 4, Figure 2), which allowed a practical MCT assay based on sequencing to be developed. Several pairs of primers were designed to amplify a 1001 bp segment within the *cpsA-cpsB* region, based on the following considerations. The primers formed amplicons from virtually all, *S. pneumoniae* isolates (>99% of those examined); the amplicon is small enough to be amplified using normal PCR protocols; the region of interest (800bp) can be sequenced using a single reaction and the method is objective. The target included most of the variable sites (bp 951 to 1747), providing maximum discrimination between closely related serotypes (e.g. members of serogroups 33 and 37 that could not be distinguished by serotype/group-specific PCR).

Table 6. Algorithm for *S. pneumoniae* molecular capsular sequence type identification by sequencing and serotype/group-specific PCR.

Amplification primer pairs*	PCR product size (base pairs)	Interpretation
<i>S. pneumoniae</i> identification primer pairs		
P1/P2	864	<i>S. pneumoniae</i>
<i>S. pneumoniae</i> mct identification by sequencing		
cpsS1/cpsA3 (for most MCT)	1001	1. Purification PCR amplicons
or	or	2. Sequencing PCR amplicons
cpsS1/cpsA1+ cpsS3/cpsA2 (for MCT 38/25F and some nontypable isolates)	520+503	3. Using programmes (Pileup & Pretty or Ednadist & Ekitsch etc.) in ANGIS to analyse sequences to identify mct/mcst
		4. Refer to Figure 1/Table 4 to identify/confirm mct/mcst.
<i>S. pneumoniae</i> mct identification by serotype/group-specific PCR		
See Table 2 for primer sequences* and Table 3 for specificity and amplicon lengths of primer pairs. Only selected molecular capsular sequence types and isolates need to be identified using the full testing algorithm.		

Some of the 376 heterogeneity sites in the *cpsA-cpsB* region were specific for individual molecular capsular sequence type (Table 4, Figure 2), while others were shared between several. Based on these patterns, plus PCR and selective sequencing of type-specific regions of *wzx* and *wzy*, most of the 51 serotypes represented among our 394 isolates could be distinguished and further divide them into a total of 71 molecular capsular sequence types, with the aid of sequence analysis software. The final CS and molecular capsular sequence typing results correlated for 343 isolates of 389 (88%) for which results for both methods were available, including five that were nontypable by either method. For 46 isolates belonging to five serotype pairs, members of which could not be distinguished by sequencing, results were classified as consistent leaving unresolved discrepancies between methods for only five (1.2%) isolates.

Sequence analysis of the *cps* gene clusters of 16 serotypes showed that *wzy* (capsular polysaccharide polymerase gene) and *wzx* (capsular polysaccharide flippase gene) are highly variable, making them suitable targets for direct serotype identification by PCR. The present inventors designed serotype-specific PCR primers for these serotypes, targeting *wzx* and *wzy* and, for serotype 3, which has no *wzy* and *wzx* genes, targeting *orf2* (*wze*)-*cap3A*-*cap3B* (Arrecubieta et al., 1996). It was found that presumed serotype-specific primers for 6A, 18C, 23F and 33F/37 were not serotype-specific, but amplified other related serotypes. To improve the molecular capsular sequence typing methods, portions of the *wzy* and *wzx* genes of serotypes within these groups were sequenced, which allowed molecular capsular sequence types to be distinguished within these serotypes/groups and demonstrate relationships between them.

The present inventors have recognized that the large number of pneumococcal serotypes would make it impractical to use serotype-specific PCR for all of them. Nevertheless, *wzy* and *wzx* PCR can be used to resolve discrepancies between CS and *cpsA-cpsB* region sequencing assays e.g. for molecular capsular sequence types 10A-23F and 23A-23F. Moreover, the use of two target regions in the *cps* gene cluster helps to clarify the relationships between mcst that have apparently arisen by recombination. Serotype/group-specific primers were evaluated using three reference panels, which had been characterised by CS and used to identify clinical isolates of unknown cs. By PCR alone, 239 (61%) of our 394 clinical isolates were assigned to a serotype or serogroup (Table 5). This method can be extended to other mct, when additional *wzx* and *wzy* sequences are available.

In some circumstances, sequencing of the *cpsA-cpsB* region may be more practical than type-specific PCR. For most serotypes only a single method and fewer primers (*cpsS1/cpsA3*-for most serotypes/isolates) are needed.

Previous studies have shown that serotypes included in 23-valent polysaccharide and 11-, 9-, 7-valent protein conjugate vaccines are those most frequently isolated from normally sterile sites (CSF, blood) (Colman et al., 1998; Huebner et al., 2000). Among 173 consecutive pneumococcal "sterile site" isolates from adults in the CIDM diagnostic laboratory, over a 2.5-year period, correlation between the mct and cs was good (171/173 CIDM isolates were correctly identified). The exceptions were two serotype 15B isolates that were identified as molecular capsular sequence type 22F. Five serotypes (4, 14, 19F, 23F, 9V –covered by all pneumococcal vaccines) accounted for 57% of isolates.

Five of 394 isolates studied were nontypable by both CS and molecular capsular sequence typing (Barker et al., 1999). Isolates may be nonserotypable because of decreased type-specific-antigen synthesis, nonencapsulated phase variation or insertion or mutation of genes of *cps* gene clusters. Failure to type them by molecular capsular sequence typing reflects the fact that the sequence database is still incomplete (also the reason for the further research in Example 2), although the target regions of two of the five nonserotypable isolates have been sequenced.

In summary, the present inventors have developed a molecular capsular sequence typing system for *S. pneumoniae*, which is reproducible, can be performed by any laboratory with access to PCR/sequencing and does not require large panels of expensive serotype-specific antisera. Work on an international collection of isolates in our reference panels demonstrated a strong correlation between the *cpsA-cpsB* sequence and CS. Heterogeneity in a relatively short sequence (800bp) in this region, supplemented by serotype/group-specific PCR targeting *wzx* and *wzy*, correctly predicted the serotype of most unknown isolates belonging to 51 serotypes. These novel molecular capsular sequence typing methods provide comprehensive strain identification that will be useful for epidemiological studies that will be needed to monitor serotype distribution and detect serotype switching, if any, among *S. pneumoniae* isolates before and following introduction and widespread use of conjugate vaccines.

EXAMPLE 2 - Identification of *S. pneumoniae* serotypes by analysis of the *wzx* and/or *wzy* genes

MATERIALS AND METHODS

Pneumococcal clinical isolates

5 This study was based on 92 well-characterized *S. pneumoniae* isolates, which represented 55 serotypes and including about 31 of 39 serotypes that were not included in Example 1. The sources of these isolates were 72 from China Medical Bacteria Culture Collection Center, Beijing, PR China; 17 from Royal College of Pathologists of Australasia, Quality Assurance Program Pty Limited, New South Wales, Australia; 10 three from Associate Professor Geoff Hogg and Ms Jenny Davis, Microbiological Diagnostic Unit (MDU), Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Victoria. Conventional serotyping (CS) had been performed by donor laboratory and serotypes of the 75 strains were known at time of receipt and 23 selected isolates (including all of serotypes 27, 28F and 16A isolates and two from Example 1 – which had been identified as one each of serotype 42 and 15 41F strains each) were re-tested by the Quellung reaction – as described above – at Department of Microbiology, Children's Hospital at Westmead (Henrichsen, 1999).

Isolates were retrieved from storage by subculture on blood agar plates (Columbia II agar base supplemented with 5% horse blood) and incubated overnight at 20 37°C in 5% CO₂.

Annotation and analysis of *wzx* and *wzy*

Analysis of homology and protein hydrophobicity was performed to annotate the *wzx* and *wzy* genes in *S. pneumoniae cps* gene cluster. Blast and PSI-blast (Altschul 25 et al., 1997) were used for searching databases including GenBank and Pfam protein motif database (Bateman et al., 2002) for possible gene functions. The TMHMM v2.0 analysis program (Chen et al., 2003) was used to identify potential transmembrane segments from the amino acid sequence. Sequence alignment and comparison were done using the program ClustalW (Thompson et al., 1994). The phylogenetic trees were 30 generated by neighbour-joining method using programme MEGA (Kumar et al. 1994) (Figures 4 and 5).

Oligonucleotide primers

In addition to our previous MCT primers (Example 1) numerous serotype(s)- 35 specific oligonucleotide primers, targeting *wzy* and *wzx* (one pair), were designed for this study. The specificity, sequences, numbered base positions and melting

temperatures (T_m) are shown in Table 7. Expected amplicon lengths of different primer pairs can be calculated from the 5'-end positions of the corresponding primers.

DNA preparation, PCR, sequencing and sequence analysis

- 5 DNA extraction, PCR, sequencing and sequence analysis were performed as described Example 1. The only exception was that, for the new PCRs, 55-60°C was used as annealing temperature because of the low T_m values of the new primers.

Nucleotide sequence accession numbers

- 10 56 new sequences generated in this study, for partial *cpsA* (*wzg*)-*cpsB* (*wzh*) genes were deposited in GenBank with accession numbers: AY508586-AY508641. These sequences form part of the present invention.

RESULTS AND DISCUSSION

15 Conventional serotyping (CS) results

- Conventional serotyping, of 23 strains, was repeated because of apparent sharing of sequence types between two or more serotypes. After careful repetitions by two different persons, a previous serotype 42 isolate was confirmed to be serotype 31 and a previous serotype 41F isolate to be serotype 41A (Example 1); serotypes of three additional isolates were also corrected. The serotypes of the other 15 isolates were confirmed to be as previously defined (including all the serotypes 27, 28F and 16A isolates, one each of serotypes 6A, 38 and 25F isolate). The final results are shown in Table 8.

25 Partial *cpsA-cpsB* sequencing primers

- The sequencing primers *cpsS1-cpsA3* produced amplicons from all strains studied in this and our previous study, except for two belonging to rare serotypes, 25F and 38, and five that were non-serotypeable (Example 1). Two additional primer pairs, *cpsS1-cpsA1* and *cpsS3-cpsA2*, formed amplicons from strains belonging to serotypes 25F and 38 and two non-serotypeable isolates.

Table 7. Oligonucleotide primers used in this study.

Name of primers	Sequence and orientation of oligo-nucleotides	Positions	T_m
10A-10B-wzy-sense	5'-TTGAGCTATTTAAGGACCTGGG-3' (SEQ ID NO:144)	395	58.4
10A-10B-wzy-antisense	3'-AGTTCTTTCACCTGCGAACGATT-5' (SEQ ID NO:145)	677	58.4
10C-10F-wzy-sense	5'-GTCAATAAGTTTAAGTGTTATAGGGC-3' (SEQ ID NO:146)	51	59.0
10C-10F-wzy-antisense	3'-CAAGCGTTGTGGGTAGTGATAT-5' (SEQ ID NO:147)	337	63.5
13-wzy-sense	5'-GATGGGAAAATACGATATGCTC-3' (SEQ ID NO:148)	427	56.1
13-wzy-antisense	3'-CGACCTCAAAACAGTACCTCAA-5' (SEQ ID NO:149)	736	58.5
20-wzy-sense	5'-CTTTATCAGGAATACGCCAATC-3' (SEQ ID NO:150)	383	56.5
20-wzy-antisense	3'-GCAACCAAGAGCAATAATATGTCC-5' (SEQ ID NO:151)	683	58.3
13-wzx-sense	5'-CTTTCTTCGTATGCTTTAGGG-3' (SEQ ID NO:152)	93	56.3
13-wzx-antisense	3'-GACTATCCACATTAGAGATAGAAGG-5' (SEQ ID NO:153)	460	53.9
20-wzx-sense	5'-GTTCTTTGTTTGACCCTTCCTT-3' (SEQ ID NO:154)	289	57.2
20-wzx-antisense	3'-TATCTTATGCGGTCTGTCGTAA-5' (SEQ ID NO:155)	604	56.4
16F-wzy-sense	5'-TTGTTCTTACATTTAGCCGTAGTG-3' (SEQ ID NO:156)	434	56.9
16F-wzy-antisense	3'-GACAGTGAGATAGTGAGTCGTTTA-5' (SEQ ID NO:157)	777	55.9
27-wzy-sense	5'-CAGAGTTTGGTCGAGGTTCCCTA-3' (SEQ ID NO:158)	455	58.7
27-wzy-antisense	3'-GAGTTAGTTGCTGCCTTTAGTG-5' (SEQ ID NO:159)	782	59.7
28F-16A-wzy-sense	5'-GATCCGCTCACGGTATGGACTA-3' (SEQ ID NO:160)	261	61.6
28F-16A-wzy-antisense	3'-GAATAACCGACTGTCGTTTTAA-5' (SEQ ID NO:161)	581	57.1
16F-wzx-sense	5'-TTTATGAGGAGAGTACTGTATCAGA-3' (SEQ ID NO:162)	1219	53.1
16F-wzx-antisense	3'-ACTCAAGCTATCGATAGTAATTTGT-5' (SEQ ID NO:163)	1433	56.6
27-wzx-sense	5'-TACATTTTTATGAGAAGAGCATTG-3' (SEQ ID NO:164)	1213	54.6
27-wzx-antisense	3'-GCTATCAGTACTATTTTTTTGTCAC-5' (SEQ ID NO:165)	1439	56.4
33A-specific-sense	5'-TTGTTGTTGGGATTGTCTTGGG-3' (SEQ ID NO:166)	length	62.1

33A-specific-antisense	3'-GTTTCAAGGCTTTAGGTTTCCG-5' (SEQ ID NO:167)	246bp	62.9
9V-specific-sense	5'- TCCTTGATTTCATCAGGGATTG-3' (SEQ ID NO:168)	length	57.0
9V-specific-antisense	3'-ATCACCATTGACGCAATCAGGA-5' (SEQ ID NO:169)	545bp	54.2
15A-15B-15C-wzx-sense	5'-ATTGCGACTGTAAACGAGAAG-3' (SEQ ID NO:170)	202	57.0
15A-15B-15C-wzx-antisense	3'-CCGTGTCTAAATACCTTTATGT-5' (SEQ ID NO:171)	514	55.0
15B-15C-wzy-sense	5'-TAATAAGCGGATGATTGTAGCG-3' (SEQ ID NO:172)	693	58.1
15B-15C-wzy-antisense	3'-GGGTAGACCTTCAATTAGTCA-5' (SEQ ID NO:173)	1041	55.5
15A-wzy-sense	5'-TATTTCTTCTATGGGACAAC-3' (SEQ ID NO:174)	840	55.6
15A-wzy-antisense	3'-CACCCTACTAATCGTAATAACA-5' (SEQ ID NO:175)	1100	54.2
22F-22A-wzy-sense	5'-AGGATGCAGTAGATACCAGTGG-3' (SEQ ID NO:176)	398	56.1
22F-22A-wzy-antisense	3'-CCTGTTGTTGGAGGCAAATATC-5' (SEQ ID NO:177)	752	56.2
22F-22A-wzx-sense	5'-GGTTCTATCAAGGAAAAGAGGAC-3' (SEQ ID NO:178)	404	56.3
22F-22A-wzx-antisense	3'-CAACCCAAGTCACTAACGATAA-5' (SEQ ID NO:179)	672	56.3
11A-specific-sense	5'-CACTTCCATATCCAGCAT-3' (SEQ ID NO:180)	727-744	47.5
11A-specific-antisense	3'-GACAGAGGACTATCAAGAGT-5' (SEQ ID NO:181)	970-989	46.4
7A-wzy-specific-sense	5'-GCAAGTGTTCATGGGAGTA-3' (SEQ ID NO:182)	76	55.3
7A-wzy-specific-antisense	3'-GAATAACATACCAGGGAGGCA-5' (SEQ ID NO:183)	420	56.1
7A-wzx-specific-sense	5'-TTTGAGAAATGCGGATAAGGTG-3' (SEQ ID NO:184)	730	58.0
7A-wzx-specific-antisense	3'-GAGTAACATTGTCCCGTTTGAA-5' (SEQ ID NO:185)	1060	56.7
11A-11D-wzy-specific-sense	5'-CGAAATATCGCCATTCATCAG-3' (SEQ ID NO:186)	190	58.4
11A-11D-wzy-specific-antisense	3'-TCACCGTGTCAACGACAATAA-5' (SEQ ID NO:187)	570	59.8
11A-11D-wzx-specific-sense	5'-CAATCAATAATGCCGCATAC-3' (SEQ ID NO:188)	856	54.3
11A-11D-wzx-specific-antisense	3'-CTAAAGCAATCAAAGGTGTCCA-5' (SEQ ID NO:189)	1140	55.6
12B-wzy-specific-sense	5'-TGGAGGAGCAACTGACGTATT-3' (SEQ ID NO:190)	518	57.3
12B-wzy-specific-antisense	3'-GAGAACTTATACCTGCCACCT-5' (SEQ ID NO:191)	783	57.5

12B-wzx-specific-sense	5'-GTATGTTATTCGTTAGACAAACTGG-3' (SEQ ID NO:192)	1058	55.6
12B-wzx-specific-antisense	3'-GACATCCAAATACATAACGCTCAA-5' (SEQ ID NO:193)	1363	56.0
17F-wzy-specific-sense	5'-CTATTACCTTGTTTCCTGCAAC-3' (SEQ ID NO:194)	490	56.1
17F-wzy-specific-antisense	3'-CTATTGCGATACAGTCGTTAAG-5' (SEQ ID NO:195)	838	54.9
17F-wzx-specific-sense	5'-GGATTACAAGAAATCCCTCG-3' (SEQ ID NO:196)	722	56.0
17F-wzx-specific-antisense	3'-TCCACTATACGCCTCGGTTAT-5' (SEQ ID NO:197)	1094	59.8
47F-wzy-specific-sense	5'-TTTGGGTCTCCTTTACCTATC-3' (SEQ ID NO:198)	725	53.2
47F-wzy-specific-antisense	3'-CACTACTTCTCAATCCCCTTT-5' (SEQ ID NO:199)	1195	53.7
25A-29-wzy-specific-sense	5'-CCGAAAATTGTTACAGGATAC-3' (SEQ ID NO:200)	112	56.8
25A-29-wzy-specific-antisense	3'-CTATACGGAACATAGGTAGTTAG-5' (SEQ ID NO:201)	474	55.9
47F-wzx-specific-sense	5'-AGCAGCAATTGTTTCTGTCTTAACA-3' (SEQ ID NO:202)	1128	60.6
47F-wzx-specific-antisense	3'-GAGATTTTCACTATCTACACTATCTT-5' (SEQ ID NO:203)	1389	52.8
25A-29-wzx-specific-sense	5'-CTCCCTATCATTACTACTCCCTATG-3' (SEQ ID NO:204)	58	56.2
25A-29-wzx-specific-antisense	3'-AATCCACGCTGTCAAGAAAGTG-5' (SEQ ID NO:205)	274	57.4
10C-10F-wzy-specific-sense	5'-GTCAATAAGTTTAAGTGTTATAGGGC-3' (SEQ ID NO:206)	51	56.2
10C-10F-wzy-specific-antisense	3'-CAAGCGTTGTGGGTAGTGATAT-5' (SEQ ID NO:207)	337	57.8
7C-wzy-sense	5'-ACTCAAGTATCTGTGC/TCACCTT-3' (SEQ ID NO:208)	453	55.7
7C-wzy-antisense	3'-CCTCGTCCATCTCCTTCACTAA-5' (SEQ ID NO:209)	703	57.1
7C-wzx-sense	5'-TGAGTTTCCGATTAGAGCAG-3' (SEQ ID NO:210)	317	53.0
7C-wzx-antisense	3'-CCTTTACTACGCCATCCATA-5' (SEQ ID NO:211)	740	54.4
9L-9N-wzy-sense	5'-TCAATGGCGACTTTATTTGC-3' (SEQ ID NO:212)	72	55.0
9L-9N-wzy-antisense	3'-CGTGGGATGTCCTCTATTATCTGA-5' (SEQ ID NO:213)	434	56.2
9L-9N-wzx-sense	5'-GTACCGCAAGCTATTCTAATGA-3' (SEQ ID NO:214)	388	54.9
9L-9N-wzx-antisense	3'-GTCATTCTATCCGCTTCAAATAG-5' (SEQ ID NO:215)	853	53.4
17A-wzy-sense	5'-TAGACTTCTTAGAGCCTATTGTGG-3' (SEQ ID NO:216)	722	55.3

17A-wzy-antisense	3'-CTGGTTATCGCGTTTGACAATA-5' (SEQ ID NO:217)	1040	56.9
17A-wzx-sense	5'-CAAACCCCTTAGTCCAATATGGCTG-3' (SEQ ID NO:218)	624	62.2
17A-wzx-antisense	3'-CCGATGGATAATAAGGGAAGCAAC-5' (SEQ ID NO:219)	988	61.0
23A-wzy-sense	5'-CATTTGGTATGGGAGTAGGGAG-3' (SEQ ID NO:220)	1049	58.1
23A-wzy-antisense	3'-GTGAAAGAGGATTGAGTACGTGG-5' (SEQ ID NO:221)	1326	58.5
33B-48-wzy-sense	5'-TAATCAA/GTGGTCTGGTGGTCA/GA-3' (SEQ ID NO:222)	453	57.9
33B-48-wzy-antisense	3'-GAAAC/TAAT/CGAGGATAACT/CGACT-5' (SEQ ID NO:223)	815	57.2
23F-wzy-sense	5'-TGTCAGCAGAAAATATGACGC-3' (SEQ ID NO:224)	402	56.4
23F-wzy-antisense	3'-CCTTTATGCTGCTTCCCAATAC-5' (SEQ ID NO:225)	766	58.4
34-wzy-sense	5'-TTGTTGTAGTGGCAGTTGCTCC-3' (SEQ ID NO:226)	740	60.4
34-wzy-antisense	3'-CGGATGTCCCTTACAGAAATGTTG-5' (SEQ ID NO:227)	1070	59.4
35A-wzy-sense	5'-TCCTGATTATG/ATTGAGATTTG/CG-3' (SEQ ID NO:228)	399	54.7
35A-wzy-antisense	3'-GACCTAACGCTTCTGAATGAAT-5' (SEQ ID NO:229)	747	54.8
36-wzy-sense	5'-CAATTTCCCCTTATTCTGTAGTTC-3' (SEQ ID NO:230)	692	56.8
36-wzy-antisense	3'-CTCTCTTGTCATATTTGTCCCAGTT-5' (SEQ ID NO:231)	1026	57.0
39(1)-wzy-sense	5'-GATTGGTTTGGGAACTTGATGTC-3' (SEQ ID NO:232)	232	60.2
39-wzy-antisense	3'-CACCATACTCCATAGTAAATCGTCC-5' (SEQ ID NO:233)	518	59.5
41A-wzy-sense	5'-GTAGTTACTGGCCCTTCTTATTCC-3' (SEQ ID NO:234)	511	59.7
41A-wzy-antisense	3'-GTTCTACGTCTATCAAAGAGCGAT-5' (SEQ ID NO:235)	828	59.0
41A-wzx-sense	5'-CAGCAAATGCAGGTTCTCAA-3' (SEQ ID NO:236)	278	59.0
41A-wzx-antisense	3'-ACTGTGGAGCAGATCGTATAGTAAT-5' (SEQ ID NO:237)	566	58.9
43-wzy-sense	5'-GATCAAATGGTGGTATTAGGAA-3' (SEQ ID NO:238)	251	54.0
43-wzy-antisense	3'-CGGTCAGTATAAAAGGTTAAGA-5' (SEQ ID NO:239)	601	55.8
43-wzx-sense	5'-TTCTTATCGCTTCCATTGTCAG-3' (SEQ ID NO:240)	907	57.5
43-wzx-antisense	3'-CCACATTCACCTCGTCGTAAA-5' (SEQ ID NO:241)	1182	57.1

47A-wzy-sense	5'-TATTTGCCATAACGGACTCTAGAAC-3' (SEQ ID NO:242)	485	59.5
47A-wzy-antisense	3'-CACCAATACACCCAAATTAAGAAGC-5' (SEQ ID NO:243)	830	61.5
47A-wzx-sense	5'-TTTGGGCTCTTTAGGTAGTGTAT-3' (SEQ ID NO:244)	687	55.4
47A-wzx-antisense	3'-CTGCCTATTACAAGCTATGAAATG-5' (SEQ ID NO:245)	1064	55.3
48-wzy-sense	5'-CATTTGGAGTTATTGCCCTAC-3' (SEQ ID NO:246)	602	54.5
48-wzy-antisense	3'-CCCCAGAATTAAATCTTATACCC-5' (SEQ ID NO:247)	909	56.6
48-wzx-sense	5'-AGGGCTTAACTGTTTCAGTGTT-3' (SEQ ID NO:248)	782	55.5
48-wzx-antisense	3'-CTAAACCATATCGTCCTGACTT-5' (SEQ ID NO:249)	1113	54.2
33C-wzy-sense	5'-TTATCTATATGTTAGGGCTG-3' (SEQ ID NO:250)	197	45.3
33C-wzy-antisense	3'-CTGTGAAGACTTACAACATG-5' (SEQ ID NO:251)	445	43.7
23B-wzy-sense	5'-TTGGATCGTTGTTTCATAGCGG-3' (SEQ ID NO:252)	639	61.0
23B-wzy-antisense	3'-GACACCTTTACGGCAACGATTC-5' (SEQ ID NO:253)	947	62.5
23B-wzx-sense	5'-AGCGAGCGGTATCATTCTATTTG-3' (SEQ ID NO:254)	897	60.8
23B-wzx-antisense	3'-CTATCACAACCTTCTTTAACGAGGTC-5' (SEQ ID NO:255)	1219	59.6
24B-wzy-sense	5'-TCAACACTTATGATGGTGCCTG-3' (SEQ ID NO:256)	685	58.5
24B-wzy-antisense	3'-ATCTTCACCCTAATAGCCCGA-5' (SEQ ID NO:257)	1025	58.3
25F-38-wzy-sense	5'-AATCTGAGGAACTTGGAGCAA-3' (SEQ ID NO:258)	641	58.5
25F-38-wzy-antisense	3'-GCATAATTGCTAATCTTAACAAGG-5' (SEQ ID NO:259)	977	55.8
25F-38-wzx-sense	5'-GCAATGGTTTATGGATGATAGAGCG-3' (SEQ ID NO:260)	702	64.3
25F-38-wzx-antisense	3'-TGTGCTGCTAACGACCACGAAA-5' (SEQ ID NO:261)	1088	64.4
31-wzy-sense	5'-TGAAAATCCCTTAGTGACATCTG-3' (SEQ ID NO:262)	492	56.5
31-wzy-antisense	3'-GACCAGCATCGTAAAGAGTCTA-5' (SEQ ID NO:263)	794	56.5
32A-32F-wzy-sense	5'-CGGTATGCTTACAATGAGACGC-3' (SEQ ID NO:264)	813	60.2
32A-32F-wzy-antisense	3'-GTAGAATAGGCCCTTGCTTAAG-5' (SEQ ID NO:265)	1163	60.5
32A-32F-wzx-sense	5'-GTAACGATGCCTAGAAATGACTT-3' (SEQ ID NO:266)	799	53.6

32A-32F-wzx-antisense	3'-CACACCATTATCCACGACAATAG-5' (SEQ ID NO:267)	1107	53.9
35B-wzy-sense	5'-CTAATTGGCTATGAAGCTAATCCC-3' (SEQ ID NO:268)	626	60.6
35B-wzy-antisense	3'-CAAATGACTGACGCTGAAATCACTT-5' (SEQ ID NO:269)	1019	58.2
45-wzy-sense	5'-CTATGCAGGAAATATCCGAGAAGG-3' (SEQ ID NO:270)	111	61.7
45-wzy-antisense	3'-GTATCGCAAAGACAAAGTGCCTAG-5' (SEQ ID NO:271)	497	63.0
45-wzx-sense	5'-AATGGCTTGCTCCTATTGCTGT-3' (SEQ ID NO:272)	929	60.9
45-wzx-antisense	3'-CGTTTAGCAAGAACCCTATCATC-5' (SEQ ID NO:273)	1306	58.1
41F-wzx-sense	5'-GTCAAAGACAGGAATGACATCTATG-3' (SEQ ID NO:274)	493	57.7
41F-wzx-antisense	3'-CCCTCCTTCACGAAAATAAAGA-5' (SEQ ID NO:275)	972	56.9
18A-18-B-18C-18F-wzx-sense	5'-GGAATCGGACAATAGCAC-3' (SEQ ID NO:276)	35	50.2
18A-18-B-18C-18F-wzx-antisense	3'-ACCAGAACTTCTCAAAGCAT-5' (SEQ ID NO:277)	265	50.5
19B-19C-wzx-sense	5'-GGCATCAAAGGTAAAGTG-3' (SEQ ID NO:278)	744	48.0
19B-19C-wzx-antisense	3'-GAAGACAGCGTTGAGAAA-5' (SEQ ID NO:279)	1171	47.5
19F-wzx-sense	5'-GCTATCTAACATTGCGAGTA-3' (SEQ ID NO:280)	672	48.4
19F-wzx-antisense	3'-AAACCGAAGGACGAATAT-5' (SEQ ID NO:281)	967	49.1
2-wzx-sense	5'-TAGCGGTGAATGGCATCT-3' (SEQ ID NO:282)	644	54.1
2-wzx-antisense	3'-AGTTGGAATCATCCTCGCT-5' (SEQ ID NO:283)	1012	50.6
23A-23F-wzx-sense	5'-GGGAAATGGTTTACTATGC-3' (SEQ ID NO:284)	623	49.7
23A-23F-wzx-antisense	3'-GTTCTTCTATTCTCGCC(T)A-5' (SEQ ID NO:285)	843	47.0
6A-6B-wzx-sense	5'-ATTTATGAAGGGAAGATGG-3' (SEQ ID NO:286)	1003	49.0
6A-6B-wzx-antisense	3'-CCGAGCGTCATTATCAAA-5' (SEQ ID NO:287)	1324	47.6
8-wzx-sense	5'-TATGTTTCAAGGGTTCTG-3' (SEQ ID NO:288)	88	45.2
8-wzx-antisense	3'-CCTTACCGTCGAATAATA-5' (SEQ ID NO:289)	356	47.4
9A-9V-wzx-sense	5'-TGATAAGGCTTACCAGTT-3' (SEQ ID NO:290)	732	44.6
9A-9V-wzx-antisense	3'-CTGACCATAACCCTGATT-5' (SEQ ID NO:291)	1360	44.0

12F-12B-44-46-wzy-sense	5'-TGAATATGGACGGTGGAG-3' (SEQ ID NO:292)	767	51.1
12F-12B-44-46-wzy-antisense	3'-GAAAGCCGAAAGAAACGA-5' (SEQ ID NO:293)	1008	53.1
14-wzy-sense	5'-GATTGGCTGTTCAAGTGT-3' (SEQ ID NO:294)	230	47.3
14-wzy-antisense	3'-CCCTGCCTAAATGTAATC-5' (SEQ ID NO:295)	463	47.2
16F-wzy-sense	5'-TTGTTCTTACATTTAGCCGT-3' (SEQ ID NO:296)	434	50.6
16F-wzy-antisense	3'-CCCTGAACCTAAACCATT-5' (SEQ ID NO:297)	737	49.9
18A-18-B-18C-18F-wzy-sense	5'-CATGAAGTTGCACCTATT-3' (SEQ ID NO:298)	409	45.2
18A-18-B-18C-18F-wzy-antisense	3'-CCCTATCCCAAACATTGT-5' (SEQ ID NO:299)	840	47.2
19F-wzy-sense	5'-AAACGGAAAGTTGGATGG-3' (SEQ ID NO:300)	667	52.8
19F-wzy-antisense	3'-CAGAAACGACATCCACGAA-5' (SEQ ID NO:301)	1075	49.9
2-3-wzy-sense	5'-TGTCGGCATTGTATTCTTTA-3' (SEQ ID NO:302)	59	51.9
2-3-wzy-antisense	3'-CCCAGTCCTAAACCACCA-5' (SEQ ID NO:303)	855	54.4
37-33F-33A-wzy-sense	5'-TAGGGAAATGGGCGACTC-3' (SEQ ID NO:304)	101	55.4
37-33F-33A-wzy-antisense	3'-ACCTCAAACCATAACTCGGA-5' (SEQ ID NO:305)	596	54.7
6A-6B-wzy-sense	5'-ATTCCAGCGACTACACTT-3' (SEQ ID NO:306)	496	46.7
6A-6B-wzy-antisense	3'-AATCACCACCATCTAACG-5' (SEQ ID NO:307)	634	45.2
8-wzy-sense	5'-CACGCAGACTAGAACAGC-3' (SEQ ID NO:308)	606	48.5
8-wzy-antisense	3'-GAACCAGATACATACGCCA-5' (SEQ ID NO:309)	1055	50.5
9A-9V-wzy-sense	5'-GTTGGTTTCGACTCTTTG-3' (SEQ ID NO:310)	394	47.5
9A-9V-wzy-antisense	3'-TTTTGCGATGACTGTTAC-5' (SEQ ID NO:311)	1017	45.7
19B-19C-wzy-sense	5'-TTCGGAGATTTGTGGTAT-3' (SEQ ID NO:312)	478	47.5
19B-19C-wzy-antisense	3'-AGCAAATACCTCCACCTA-5' (SEQ ID NO:313)	772	50.0
1-wzx-sense	5'-TGGAGAATTTGCGATTACG-3' (SEQ ID NO:314)	744	54.5
1-wzx-antisense	3'-TAGAGTCCCATTGTCTCAC-5' (SEQ ID NO:315)	886	48.6
4-wzx-sense	5'-AATGCTTGTACTIONCCCTC-3' (SEQ ID NO:316)	88	48.5

4-wzx-antisense	3'-GATACTAAATGCCTACCG-5' (SEQ ID NO:317)	898	48.1
19A-wzx-sense	5'-TTCCCTATGTCAGTCTATGAA-3' (SEQ ID NO:318)	1000	49.7
19A-wzx-antisense	3'-TCTTCATAGTATCGGCTTAA-5' (SEQ ID NO:319)	1214	48.8
1-wzy-sense	5'-TATTCTATTTCTTACCCGCTAC-3' (SEQ ID NO:320)	211	51.6
1-wzy-antisense	3'-ATTCACCCGTTCAAAGTAGA-5' (SEQ ID NO:321)	801	52.4
4-wzy-sense	5'-GTGCCTAGTAGCATTCCATA-3' (SEQ ID NO:322)	1003	50.5
4-wzy-antisense	3'-GAAACCAATGATACCACCAC-5' (SEQ ID NO:323)	1198	50.4
19A-wzy-sense	5'-TCGCCTAGTCTAAATACCAA-3' (SEQ ID NO:324)	235	50.7
19A-wzy-antisense	3'-AAGTGAATCTTAAAGCCGTC-5' (SEQ ID NO:325)	975	53.4
17F-YS2-sense	5'-AGAGGGATTGTTGAAGGTATTC-3' (SEQ ID NO:326)	754	59.8
17F-YA2-antisense	3'-CCTACTATCTTTACGCTCTGAT-5' (SEQ ID NO:327)	1060	59.7
25F-38-YS-sense	5'-GGCGTTGTCAGTGCTAGTTTAG-3' (SEQ ID NO:328)	121	62.6
25F-38-YA-antisense	3'-CTCATATTACCGACGAAATTGTCC-5' (SEQ ID NO:329)	713	61.6
35F-47F-YS-sense	5'-ATAAAAAGAAAGTCTTTGCCAGAG-3' (SEQ ID NO:330)	13	60.6
35F-47F-YA-antisense	3'-CTACTACTTGTATCAGCGATAAC-5' (SEQ ID NO:331)	499	60.0
25A-29-YS-sense	5'-CCGAAAATTGTTACAGGATAC-3' (SEQ ID NO:332)	112	62.0
25A-29-YA-antisense	3'-CTATACGGAACATAGGTAGTTAG-5' (SEQ ID NO:333)	474	60.9

Updated sequence type nomenclature (compared with Example 1)

- Sequence types were generally named according to the corresponding serotype, with a suffix representing the source of the isolate for which the sequence type was first identified. When sequences characteristic of two to five serotypes were identified, the sequence type name included all, with the lower number serotype first (e.g 15B-15C-22F-22A etc.) (Henrichsen, 1995). Representative sequences of all sequence types were deposited into GenBank (see Table 8 for sequence type nomenclature and corresponding GenBank accession numbers).

Table 8. *S. pneumoniae* partial *cpsA-cpsB* sequence (~800 bp) database and comparison of molecular capsular typing (MCT) and conventional serotyping (CS) results of 519 *S. pneumoniae* isolates (and also including 24 GenBank sequences and 92 Sanger Institute sequences).

CS ^a	Sequence types (n=) ^{ab}	GenBank (positions) ^c	No. Serotype/group-specific (n=) ^a	PCR	Final MCT (n=) ^{ab}	Comments ^{ad}
1	1-g (g)	Z83335 (4545-5343)	1 (1)		1 (1)	Correlate
	1-q (9+1A)	AF532632	1 (9+1A)		1 (9+1A)	Correlate
	2-g (g)	AF026471 3210)	2 (1)		2 (1)	Correlate
3	2-q (3)	AF532669	2 (3)		2 (3)	Correlate
	2-41A (s)	20602 (2612-3410)	2 (1)		2 (1)	Correlate
	3-g (g)	Z47210 (2413- 3210)	3 (1)		3 (1)	Correlate
	3-q (15+qap)	AF532682	3 (16)		3 (16)	Correlate
	3-c (1)	AF532681	3 (1)		3 (1)	Correlate
4	3-nz (1)	AF532683	3 (1)		3 (1)	Correlate
	4 (gx2+36+qap)	AF316639 (2470- 3268), NC_003028 (genome); AF532693	4 (39)		4 (39)	Correlate
5	5-q (4)	AF532697	NA		5 (4)	Correlate
	5-c (1)	AF532696	NA		5 (1)	Correlate
	5-qap (qap)	AY508634	NA		5 (1)	Correlate

6A	6A-g (g)	AY078347 1967)	(1169-	Serogroup 6 (1)	6 (1)	Correlate
	6A-c1 (2)	AF532699		Serogroup 6 (2)	6A (2)	Correlate
	6A-c2 (1)	AF532700		Serogroup 6 (1)	6A (1)	Correlate
	6A-n (2)	AF532698		Serogroup 6 (2)	6A (2)	Correlate
	6A-qap (qap)	AY508641		Serogroup 6 (1)	6A (1)	Correlate
	6A-6B-g (1)	AF532701		Serogroup 6 (1)	6A or 6B (1)	Consistent
	6A-6B-q (1)	AY330713		Serogroup 6 (1)	6A or 6B (1)	Consistent
	6A-6B-s (5+s)	AF532702/ 17611 (2259-3057)		Serogroup 6 (6)	6A or 6B (6)	Consistent
6B	6B-c (1)	AF532704		Serogroup 6 (1)	6B (1)	Correlate
	6A-6B-g (g+5)	AF316640 2952); AF532703	(2154-	Serogroup 6 (6)	6A or 6B (6)	Consistent
	6A-6B-q (9)	AF532705		Serogroup 6 (9)	6A or 6B (9)	Consistent
	6A-6B-s (s)	17506 (2157-2955)		Serogroup 6 (1)	6A or 6B (1)	Consistent
7F	7F-7A (15+qap+s)	AF532707/ 20024 (2531-3329)		NA	7F or 7A (17)	Consistent
7A	7A-cn (cn)	AY508635		NA	7A (1)	Correlate
	7F-7A (s)	24019 (2502-3300)		NA	7F or 7A (1)	Consistent
7B	7B-40(cnx2)	AY508636, AY508627		7B or 7C or 40 (2)	7B or 40 (2)	Consistent
7C	7C-19C-24B (7+s)	AF532706/ 21759 (2804-3602)		7B or 7C or 40 (8)	7C (8)	Correlate

8	8-g (gx2+12)	AF31664 (2511-3309), AF532708	8 (14)	Correlate
	8-s (s)	13844 (2518-3316)	8 (1)	Correlate
9A	9A-9V (cn+s)	AY508637/ 20538 (2486-3284)	9A or 9V (2)	Consistent
9L	9L-cn (cn+s)	AY508638/ 17618 (2805-3603)	9L (2)	Correlate
9N	9N (9+s)	AF532709/ 17619 (2805-3603)	9N (10)	Correlate
9V	9V (g+17)	AF402095 (1520-2318); AF532710	9V (18)	Correlate
	9A-9V (cn+s)	AY508639/ 20856 (2803-3601)	9A or 9V (2)	Consistent
9V and 14	9V (1)	AF402095 (1520-2318); AF532710	9V and 14 (1)	Correlate
10F	10F-q (3)	AF532635	10F (3)	Correlate
	10F-ca (2)	AF532636	10F (2)	Correlate
	10F-10C (qap+s)	AY508587/ 18532 (2201-2999)	10F or 10C (2)	Consistent
10A	10A-17A (5+cn+s)	AF532633/ 17290 (2451-3249)	10A (7)	Correlate
	10A-23F (6)	AF532634	10A (6)	Correlate

10B	10B-10C (cn+s)	AY508586/ 16991 (2154-2952)	10A or 10B (2)	10B (2)	Correlate
10C	10F-10C (s)	18126 (2095-2893)	10F or 10C (1)	10F or 10C (1)	Consistent
11F					
11A	11A-nz (1)	AF532638	11A or 11D (1)	11A (1)	Correlate
	11A-11D-18F (7+s)	AF532637/ 17948 (3506-4304)	11A or 11D (8)	11A or 11D (8)	Consistent
11B	11B-11C (1+cn)	AF532639	NA	11B or 11C (2)	Consistent
11C	11B-11C (cn)	AY508588	NA	11B or 11C (1)	Consistent
	11C-cn (cn)	AY508589	NA	11C (1)	Correlate
11D	11A-11D-18F (s)	17213 (2852-3650)	11A or 11D (1)	11A or 11D (1)	Consistent
12F	12F-q (8+1B)	AF532640	Serogroup 12 or 44 or 46 (8+1B)	12F (8+1B)	Correlate
	12F-12A-12B (1+s)	AF532641/ 23778 (2154-2952)	Serogroup 12 or 44 or 46 (2)	12F or 12A or 12B (2)	Consistent
12A	12A-cn (cn)	AY508590	Serogroup 12 or 44 or 46 (1)	12A (1)	Correlate
	12A-46 (s)	27104 (4224-5022)	Serogroup 12 or 44 or 46 (1)	12A or 46 (1)	Consistent
	12F-12A-12B (cnx2)	AY508591	Serogroup 12 or 44 or 46 (2)	12F or 12A or 12B (2)	Consistent
12B	12F-12A-12B (s)	23673 (2153-2951)	Serogroup 12 or 44 or 46 (1)	12F or 12A or 12B (1)	Consistent
13	13-20 (6+s)	AF532642/ 17717 (2486-3284)	13 (7)	13 (7)	Correlate
14	14-g (g)	X85787 (2369-3167)	14 (1)	14 (1)	Correlate
	14-q (23+1C)	AF532643	14 (23+1C)	14 (23+1C)	Correlate

14-v (9+s)	AF532644/ 19918 (2150-2948)	14 (10)	14 (10)	Correlate
14-c (1)	AF532645	14 (1)	14 (1)	Correlate
15F	AY508594/ 22405 (2503-3301)	15F or 15A (3)	15F (3)	Correlate
15F-cn2 (cn)	AY508595	15F or 15A (1)	15F (1)	Correlate
15A-ca1 (1+v)	AF532646	15F or 15A (2)	15A (2)	Correlate
15A-ca2 (3+s)	AF532647/ 18517 (2152-2950)	15F or 15A (4)	15A (4)	Correlate
15B-c (1)	AF532648	15B or 15C (1)	15B (1)	Correlate
15B-15C (6)	AF532649	15B or 15C (6)	15B or 15C (6)	Consistent
15B-15C-22F-22A (2+s)	AF532650/ 18624 (2154-2952)	15B or 15C (3)	15B or 15C (3)	Consistent
15C-ca (1)	AF532652	15B or 15C (1)	15C (1)	Correlate
15C-q1 (1)	AF532651	15B or 15C (1)	15C (1)	Correlate
15C-q2 (2)	AY330714	15B or 15C (2)	15C (2)	Correlate
15C-q3 (1)	AY330715	15B or 15C (1)	15C (1)	Correlate
15C-s (s)	18262 (2154-2952)	15B or 15C (1)	15C (1)	Correlate
15B-15C (v)	AY508593	15B or 15C (1)	15B or 15C (1)	Consistent
15B-15C-22F-22A (v)	AY508592	15B or 15C (1)	15B or 15C (1)	Consistent
16F-q (5+cn+s)	AF532653/ 21481 (2508-3306)	NA	16F (7)	Correlate
16F-nz (1)	AF532654	NA	16F (1)	Correlate

16A	16A-28F (cn+s)	AY508596/ 21730 (2147-2945)	16A (2)	16A (2)	Correlate
17F	17F-n (3+s+1D)	AF532656/ 22896 (2489-3287)	17F-n (4+1D)	17F (4+1D)	Correlate
	17F-35B-35C-42 (2)	AF532657	17F (2)	17F (2)	Correlate
17A	17A-ca (1+s)	AF532655/ 23198 (1645-2443)	17A (2)	17A (2)	Correlate
	10A-17A (cn)	AY508597	17A (1)	17A (1)	Correlate
18F	18F-ca (1)	AF532662	Serogroup 18 (1)	18F (1)	Correlate
	18F-w (1)	AY330716	Serogroup 18 (1)	18F (1)	Correlate
	11A-11D-18F (cn+s)	AY508598/ 22849 (2530-3328)	18F (2) ^e	18F (2)	Correlate
18A	18A-nz (5+qap+s)	AF532659/ 21887 (2247-3045)	Serogroup 18 (7)	18A-nz (7)	Correlate
	18A-q (1)	AF532658	Serogroup 18 (1)	18A (1)	Correlate
18B	18B-18C (4+s)	AF532660/ 21819 (2153-2951)	Serogroup 18 (5)	18B or 18C (5)	Consistent
18C	18B-18C (g+14+s)	AF316642 (2052- 2850); AF532661/ 21819 (2153-2951)	Serogroup 18 (16)	18B or 18C (16)	Consistent

19F	19F-g1 (gx4+7+s)	AF030367 (4724-5522), AF030368, AF030370, AF030371; AF532667/19798 (4425-5223)	19F (12)	Correlate
	19F-g2 (gx2)	AF030369 (2455-3253), AF030372	19F (2)	Correlate
	19F-g3 (g)	U09239 (1119-1917)	19F (1)	Correlate
	19F-q (9)	AF532666	19F (9)	Correlate
	19F-n (3)	AF532668	19F (3)	Correlate
	19F-c (1)	AF532665	19F (1)	Correlate
19A	19A-g (g)	AF094575 (2683-3481)	19A (1)	Correlate
	19A-q (8)	AF532663	19A (8)	Correlate
	19A-ca (3)	AF532664	19A (3)	Correlate
19B	19B-cn (cnx3+s)	AY508599/21568 (2171-2969)	19B (4)	Correlate
19C	19C-cn1 (cn)	AY508600	19C (1)	Correlate
	19C-cn2 (cnx2)	AY508601	19C (2)	Correlate
	7C-19C-24B (s)	25632 (4069-4867)	19C (1)	Correlate

20	13-20 (8+s)	AF532670/ 20453 (2486-3284)	20 (9)	20 (9)	Correlate
21	21-ca (1)	AF532671	NA	21 (1)	Correlate
	21-cn (cn)	AY508602	NA	21 (1)	Correlate
22F	15B-15C-22F-22A (13+qap+s)	AF532673/ 22696 (2486-3284)	22F or 22A (15)	22F or 22A (15)	Consistent
22A	22A (4)	AF532672	22F or 22A (4)	22A (4)	Correlate
	15B-15C-22F-22A (s)	22591 (2486-3284)	22F or 22A (1)	22F or 22A (1)	Consistent
23F	23F-c (1)	AF532678	23F (1)	23F (1)	Correlate
	10A-23F (gx3+18+s)	AF057294 (2991- 3789), AF030373, AF030374; AF532677/ 22330 (2852-3650)	23F (22)	23F (22)	Correlate
23A	23F-23A (1)	AF532679	23F (1)	23F (1)	Correlate
	23A-ca (3+s)	AF532675/ 21475 (2154-2952)	23A (4)	23A (4)	Correlate
23B	23F-23A (1)	AF532674	23A (1)	23A (1)	Correlate
	23B-c (2+s)	AF532676/ 23047 (3537-4335)	NA	23B (3)	Correlate
24F	23B-q (2)	AY330717	NA	23B (2)	Correlate
	24F-cn1 (cn)	AY508605	NA	24F (1)	Correlate
	24F-cn2 (cn)	AY508606	NA	24F (1)	Correlate

	24F-cn3 (cn)	AY508607	NA	24F (1)	Correlate
24A	24A-cn (cn)	AY508603	NA	24A (1)	Correlate
24B	7C-19C-24B (cn+s)	AY508604/ 23976 (2534-3332)	24B (2) ^g	24B (2)	Correlate
25F	25F-38 (1+cn+s)	AF532711/ 28389 (9131-9922)	25F or 38 (3)	25F or 38 (3)	Consistent
25A	25A-29 (s)	15096 (2153-2951)	NA	25A or 29 (1)	Consistent
27	?27-28F-28A (s)	22978 (2486-3284)	27 or 28A (1)	27 or 28A (1)	Consistent
	27-cn (cnx4)	AY508608	NA	27 (4)	Correlate
28F	16A-28F (s)	21731 (2147-2945)	16A or 28F (1)	16A or 28F (1)	Consistent
	?27-28F-28A (cnx3)	AY508610	28F or 28A (3)	28F or 28A (3)	Consistent
	28F-cn (cn)	AY508611	28F or 28A (1)	28F (1)	Correlate
28A	?27-28F-28A (cnx3+s)	AY508609/ 22978 (2486-3284)	28F or 28A (4)	28F or 28A (4)	Consistent
29	29-ca (1)	AF532680	NA	29 (1)	Correlate
	25A-29 (3+s)	AY330718/ 15096 (2153-2951)	NA	25A or 29 (4)	Consistent
31 ^h	31 (6+1 ^h +s+1E)	AF532684, AF532695/ 22164 (2538-3336)	31 (6+1 ^h +s+1E)	31 (8+1E)	Correlate
32F	32F-32A (cn+s)	AY508614/ 25372 (5428-6226)	NA	32F or 32A (2)	Consistent
32A	32A-cn (cn)	AY508613	NA	32A (1)	Correlate

	32F-32A (cn+s)	AY508612/ 25363 (5327-6125)	NA	32A or 32F (2)	Consistent
33F	33F-g (g)	AJ006986 (2483- 3281)	33F or 33A or 37 (1)	33F (1)	Correlate
	33F-q (1)	AF532687	33F or 33A or 37 (1)	33F (1)	Correlate
	33F-33B (3)	AF532688	33F or 33A or 37 (3)	33F (3)	Correlate
	33F-33A-35A (2+s)	AF532689/ 16989 (2155-2953)	33F or 33A or 37 (3)	33F or 33A (3)	Consistent
33A	33F-33A-35A (1+cn+s)	AF532685/ 18409 (2155-2953)	33F or 33A or 37 (3)	33F or 33A (3)	Consistent
33B	33B-q (3+gap)	AF532686	33B or 33C or 33D (4)	33B (4)	Correlate
	33B-s (s)	19039 (2508-3306)	33B or 33C or 33D (1)	33B (1)	Correlate
	33F-33B (cn)	AY508615	33B or 33C or 33D (1)	33B (1)	Correlate
33C	33C-s (s)	15918 (2155-2953)	33B or 33 C or 33D (1)	33C (1)	Correlate
	33C-cn (cn)	AY508616	33B or 33C or 33D (1)	33C (1)	Correlate
33D	33D-48 (s)	17583 (2508-3306)	33B or 33C or 33D (1)	33D or 48 (1)	Consistent
34	34-ca (4+gap)	AF532690	NA	34 (5)	Correlate
	34-s (s)	15938 (2425-3223)	NA	34 (1)	Correlate
35F	35F-47F (6+s)	AF532692/ 15137 (2807-3605)	35F or 47F (7)	35F or 47F (7)	Consistent
35A	33F-33A-35A (cn+s)	AY508617/ 21463 (2200-2998)	35A or 35C or 42 (2)	35A (2)	Correlate

35B	17F-35B-35C-42 (9+s)	AF532691/ 16658 (2186-2984)	35B (10)^f	35B (10)^f	Correlate
35C	17F-35B-35C-42 (cnx2+s+qap)	AY508618, AY508640/ 19741 (2518-3316)	35C or 42 (4)^f	35C or 42 (4)^f	Consistent
36	36-cn (cnx2+s)	AY508619/ 19113 (2805-3603)	NA	36-s (3)	Correlate
37	37-g (g)	AJ131984 (2849- 3648)	33F or 33A or 37 (1)	37 (1)	Correlate
38	37-ca (1+cnx2+qap+s)	AF532713/ 17777 (2557-3355)	33F or 33A or 37 (5)	37 (5)	Correlate
39	25F-38 (7+s)	AF532712/ 30298 (10688-11479)	25F or 38 (8)	25F or 38 (8)	Consistent
40	39-cn (cn+s)	AY508620/ 17810 (2202-3000)	NA	39 (2)	Correlate
41F	39-cn (cn)	AY508621	NA	39 (1)	Correlate
41Aⁱ	7B-40(cn+s)	AY508622/ 22089 (2833-3631)	7C or 40 (2)	40 (2)	Consistent
	41F-cn (cn)	AY508624	41F-wzx (1)	41F (1)	Correlate
	41F-s (s)	22917 (2848-3646)	41F-wzx (1)	41F (1)	Correlate
	2-41A (1ⁱ+cn+s)	AY508623, AF532694 ^m / 22520 (2554-3352)	(41F-wzx)41A (3)	41A (3)	Correlate

42	17F-35B-35C-42 (cn+s)	AY508625/ 19403 (2387-3185)	35A or 35C or 42 (2) ^f	35B or 35C or 42 (2) ^f	Consistent
43	43-cn (cnx2+s)	AY508626/ 22097 (2018-2816)	NA	43 (3)	Correlate
44	44-s (s)	24095 (2181-2979)	Serogroup 12 or 44 or 46 (1)	44 (1)	Correlate
45	45-cn (cn+s)	AY508628/ 27591 (2540-3338)	NA	45 (2)	Correlate
46	46-s (s)	25070 (2186-2984)	Serogroup 12 or 44 or 46 (1)	46 (1)	Correlate
47F	12A-46 (cnx2)	AY508629	Serogroup 12 or 44 or 46 (1)	12A or 46 (2)	Consistent
	35F-47F (cn+s)	AY508631/ 16064 (2538-3336)	35F or 47F (2)	35F or 47F (2)	Consistent
47A	47A-cn (cn+s)	AY508630/ 17250 (2535-3333)	NA	47A (2)	Correlate
48	48-cn (cn)	AY508633	NA	48 (1)	Correlate
48(1)	33D-48 (s)	17583 (2508-3306)	33B or 33 C or 33D or 48(1)	33D or 48 (1)	Consistent
	48(1)-cn (cn+s)	AY508632/ 22062 (2372-3170)	NA	48(1) (2)	Correlate
NT	NT-nz (1) ^j	AF532714	NA	NT (1) ^e	Correlate
	NT-ca (1) ^j	AF532715	NA	NT (1) ^e	Correlate
	NT (3) ^j	NA	NA	NT (3) ^e	Correlate

Notes.

- a. Bold letter/numbers indicate results "consistent" (see below for their definition) between MCT and CS; limited CS is needed to distinguish 2-5 serotypes within sequence types, also see text for further explanations. NT=nonserotypeable or nontypeable. Figures in parentheses indicate number of isolate and strain source for the 87 strains used in the study, the GenBank and Sanger Institute strains were also calculated into the total numbers.
- b. For explanation of sequence type nomenclature, see text. Key: -g (GenBank sequence); -c (CIDM); -n (New South Wales); -q (Queensland); -w (Western Australia); -v (Victoria); -ca (Canada); -nz (New Zealand); -cn (China); -qap (QAP programme); -s (Sanger). Different serotypes/sequence types that share the same sequences are bolded.
- c. GenBank sequence accession numbers for corresponding sequence type: Those before "," are described by the others, one sequence start and stop positions corresponding the ~800 bp regions were given; those behind "," are the sequences we studied; the sequence behind "/" were got from Sanger Institute Streptococcus pneumoniae capsular loci sequence project sequence start and stop positions corresponding the ~800 bp regions are given.
- d. "Correlate" means that MCT and CS results were identical; "consistent" means that components of MCT results (sequence type or PCR) correlated with more than one (2-5) CS result.
- e. Serogroup 18 PCR positive and 11A-11D specific PCR negative, which can confirm the strains would be 18F.
- f. Serotype 17F PCR negative.
- g. 7C and 19B-19C PCR negative, 24B could be identified by exclusion.
- h. One previous 42 strain (Example 1) was finally proved to be 31 – after twice repeat conventional serotyping and serotype 31-specific PCR positive.
- i. One previous 41F strain (Example 1) was finally proved to be 41A – after twice repeat conventional serotyping.
- j. Some of these isolates may belong to rare sequence types or even serotypes (other than the known 90 serotypes) not represented among our reference isolates.

Are the shared sequence types plausible?

In order to explain the many shared sequence types, we studied their antigenic formula (Henrichsen, 1995). Among the 31 shared sequence types (Table 9), six were shared between unrelated serotypes (2-41A, 10A-17A, 10A-23F, 13-20, 25A-29, 33D-48), three were shared between two to three related and at least another unrelated serotype (7B-40, 11A-11D-18F, 27-28F-28A, 17F-35B-35C-42) and 20 were shared between antigenically related serotypes. The remaining shared sequence type involved serotypes 16A and 28F; although they are not directly related, 28F is related to serogroup 16 (Table 9) (Henrichsen, 1995). Thus most shared molecular capsular or sequence types (genotypes) involve closely related serotypes (or phenotypes). The 10 shared sequence types that involve unrelated or more distantly related (such as 16A-28F) serotypes probably can be explained by recombination events between serotypes.

Are *wzx* and *wzy* helpful?

In Example 1 it was shown that *wzy* and *wzx* based PCRs increase the accuracy of *cpsA-cpsB* sequence-based serotype prediction. Thus, in order to extend our serotype-prediction strategy to all 90 serotypes, we examined the *wzx* and *wzy* sequences of the 90 serotypes, especially the 31 shared sequence types (Tables 7 and 9). In addition to the sequences we have determined, the unannotated sequences from the *cps* gene clusters of all 90 serotypes as determined by the Sanger Institute was used to determine the 90 *wzx* and *wzy* sequences. The identical of suitable serotype-specific *wzx* and *wzy* based primers was far from straightforward. For most of the 90 serotypes, *wzy* is shorter but more heterogeneous than *wzx* and therefore a more suitable single target for serotype-specific PCR. The *wzy* sequencing results showed that it would be helpful for the discrimination of 7C-40, 10F-10C, 12A/46 (identical)-12F/12B/44 (identical), 35A-35C/42 (identical), 35F-47F serotype(s) pairs.

It is shown that *wzx* genes from 28 different serotypes share high-level homology (72% to 100%). We found three main recombination sites in these 28 *wzx* (base positions 395, 775 and 1150) using the programme PhylPro 1.0 (Weiller 1998), which generated the diagrammatic representation of polymorphic sites and hypothetical recombination events of the *wzx* gene shown in Figure 6.

Table 9. The relationship between shared *S. pneumoniae* partial *cpsA-cpsB* sequence (798-800 bp) type and conventional serotyping (CS) antigenic formulas.

Involved CS ^a	Involved sequence types ^b	Antigenic formulas ^{cd}	wzx identity (%) ^e	wzy identity (%) ^e	Selected PCR ^f	cps gene cluster (%) ^g
2	2-41A	2a (NCR)	-	-	2	-
41A		41a (NCR)	SD	SD	41F-41A	-
6A	6A-6B-g, 6A-6B-q, 6A-6B-s	6a, 6b	-	-	IP	-
6B		6a, 6c	99.929	99.851	IP	99.079
7F	7F-7A	7a, 7b	-	-	IP	-
7A		7a, 7b, 7c	100.000	100.000	IP	SD
7B		7a, 7d, 7e, 7h	-	-	7B-7C-40	-
40		40a, 7g, 7h	-	-	7B-7C-40	-
7C	7C-19C-24B	7a, 7d, 7f, 7g, 7h	-	-	7B-7C-40	-
19C		19a, 19c, 19f, 7h	67.002	SD	19B-19C	-
24B		24a, 24b, 24e, 7h	98.903	SD	24B	-
9A	9A-9V	9a, 9c, 9d	-	-	IP	-
9V		9a, 9c, 9d, 9g	100.000	100.000	IP	99.990 (except beginning)
10F	10F-10C	10a, 10b	-	-	Seq-wzy	-
10C		10a, 10b, 10c, 10f (NCR)	99.293	98.801	Seq-wzy	96.970
10A	10A-17A	10a, 10c, 10d (NCR)	-	-	10A-10B	-
17A		17a, 17c (NCR)	SD	SD	17A	-
10A	10A-23F	10a, 10c, 10d (NCR)	-	-	10A-10B	-
23F		23a, 23b, 18b (NCR)	SD	SD (>wzx)	23F	-
10B	10B-10C	10a, 10b, 10c, 10d, 10e	-	-	10A-10B	-
10C		10a, 10b, 10c, 10f	83.156	95.843	10A-10B-neg& Seq-wzy	-

11A	11A-11D-18F	<u>11a</u> , <u>11c</u> , <u>11d</u> , <u>11e</u>	-	11A-11D	-
11D		<u>11a</u> , <u>11b</u> , <u>11c</u> , <u>11e</u>	100.000	11A-11D	99.393
18F		18a, 18b, 18c, 18f(NCR)	SD	sergroup18	-
11B	11B-11C	<u>11a</u> , <u>11b</u> , <u>11f</u> , <u>11g</u>	-	NA	-
11C		<u>11a</u> , <u>11b</u> , <u>11c</u> , <u>11d</u> , <u>11f</u>	-	NA	-
12F	12F-12A-12B	<u>12a</u> , <u>12b</u> , <u>12d</u>	-	Seq-wzy	-
12A		<u>12a</u> , <u>12c</u> , <u>12d</u>	99.417	Seq-wzy	SD
12B		<u>12a</u> , <u>12b</u> , <u>12c</u> , <u>12e</u>	99.741	Seq-wzy	98.965
					(12B:12A=9 6.939)
12A	12A-46	<u>12a</u> , <u>12c</u> , <u>12d</u>	-	Seq-wzy	-
46		<u>46a</u> , <u>12c</u> , <u>44b</u>	100.000	Seq-wzy	93.210
13	13-20	<u>13a</u> , <u>13b</u> (NCR)	-	13	-
20		<u>20a</u> , <u>20b</u> , <u>7g</u> (NCR)	83.828	20	-
15B	15B-15C	<u>15a</u> , <u>15b</u> , <u>15d</u> , <u>15e</u> , <u>15h</u>	-	IP	-
15C		<u>15a</u> , <u>15d</u> , <u>15e</u>	100.000	IP	99.979
15B	15B-15C-22F-22A	<u>15a</u> , <u>15b</u> , <u>15d</u> , <u>15e</u> , <u>15h</u>	-	15B-15C	-
15C		<u>15a</u> , <u>15d</u> , <u>15e</u>	100.000	15B-15C	-
22F		<u>22a</u> , <u>22b</u>	SD (55.623)	22F-22A	-
22A		<u>22a</u> , <u>22c</u>	SD (22A:22F=	22F-22A	22F:22A=99
			100.000)		.996
16A	16A-28F	<u>16a</u> , <u>16c</u> (NCR) ^h	-	IP	-
28F		<u>28a</u> , <u>28b</u> , <u>16b</u> , <u>23d</u> (NCR) ^h	100.000	IP	99.991
17F	17F-35B-35C-42	<u>17a</u> , <u>17b</u> (NCR)	-	17F	-
35B		<u>35a</u> , <u>35c</u> , <u>29b</u>	SD	35B	-
35C		<u>35a</u> , <u>35c</u> , <u>20b</u> , <u>42a</u>	SD	Seq-wzy	35C:35A=96
			(35C:35B=77.189)		.135
			(35C:35A=99.859)		
			SD		42:35C=99.8
		<u>42a</u> , <u>20b</u> , <u>35c</u>	(42:35C=100.000)	Seq-wzy	30

18B	18B-18C	18a, 18b, 18e, 18g	-	-	IP	-
18C		18a, 18b, 18c, 18e	100.000	100.000	IP	99.991
23F	23F-23A	23a, 23b, 18b	-	-	23F	-
23A		23a, 23c, 15a	99.928	SD	23A	-
25F	25F-38	25a, 25b	-	-	Seq-wzy	-
38		38a, 25b	99.506	99.581	Seq-wzy	97.378
25A	25A-29	25a, 25c, 38a (NCR)	-	-	25A-29	-
29		29a, 29b, 13b (NCR)	100.000	100.000	25A-29	100.000
27	27-28F-28A	27a, 27b (NCR)	-	-	28F-28A	-
28F		28a, 28b, 16b, 23d	SD	SD	28F-28A	-
28A		28a, 28c, 23d	100.000	100.000	28F-28A	99.991
			(28A:28F= SD)	(28A:28F= SD)		
32F	32F-32A	32a, 27b	-	-	IP	-
32A		32a, 32b, 27b	100.000	100.000	IP	99.996
33F	33F-33A-35A	33a, 33b, 33d	-	-	33F-33A-37	-
33A		33a, 33b, 33d, 20b	100.000	100.000	33F-33A-37	99.792
35A		35a, 35c, 20b	77.754	SD	35A-35C-42	-
33F	33F-33B	33a, 33b, 33d	-	-	33F-33A-37	-
33B		33a, 33c, 33d, 33f	77.951	SD	33B-33C-33D-48	-
33D	33D-48	33a, 33c, 33d, 33f, 6a (NCR)	-	-	IP	-
48		48a (NCR)	100.000	100.000	IP	99.989
35F	35F-47F	35a, 35b, 34b	-	-	Seq-wzy	-
47F		47a, 35a, 35b	99.859	99.754	Seq-wzy	94.749
						(except beginning and end)

Notes.

- a. Those conventional serotypes (CS) that could share the same sequence types.
- b. Those sequence types that could be shared by different (2-5) conventional serotypes.
- c. Bold parts showed that the factor antiserum are shared by all the shared sequence types related serotypes; underline part showed that the factor antiserum are shared by partial (2-4) shared sequence types related serotypes.
- d. NCR: no cross-reaction of any factor antiserum in the antigenic formulas between serotypes that share sequence types (Henrichsen, 1995).
- e. Sequence identity was calculated by the comparison of wzx and wzy sequences – the others wzx and wzy compared the first CS in the several sharing ST CS. SD: significant length and sequence differences (heterogeneity) between wzx or wzy.
- f. Only selected some PCR to show cases and the “serotype-specific” PCR was only evaluated within the related CS that shared sequence types. IP=impossible (or unlikely) to design real serotype-specific PCR primers to differentiate between the share sequence serotypes because the very high wzx and wzy sequence similarity.
- g. Only those with very high wzx and wzy sequence similarity serotypes cps gene cluster comparison results are shown.

Comprehensive molecular capsular sequence typing results

The final molecular capsular sequence typing results for 519 isolates (427 previously studied and 92 new isolates) are shown in Table 9. Our database now includes 90 *S. pneumoniae* serotypes and 134 sequence types (including two non-serotypeable strains). 83 serotypes are represented by 2 or more strains. 102 sequence types (not including two nonserotypeable strains), including 47 that are represented by two or more isolates, correspond to a single serotype; 23 sequence types are shared by two serotypes, six are shared by three serotypes and two are shared by four serotypes (Table 8).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

All publications discussed above are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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